AMPHIBIAN SKIN MICROBIOTA AND SURFACE PEPTIDES: DYNAMICS OF INNATE IMMUNE DEFENSE INTERACTIONS WITH THE EMERGING FUNGAL PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS*

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“Le microbe n'est rien, le terrain est tout.”

“The microbe is nothing.
It is the terrain in which it is found
that is everything.”

“Die Mikrobe ist nichts, das Milieu
ist alles.”

Louis Pasteur (1822-1895)
Antoine Béchamp (1816-1908)
Claude Bernard (1813-1878)

CONTENTS

SUMMARY

The work presented in this dissertation explores contemporary disease ecology questions framed by the looming amphibian disease, chytridiomycosis.

- How do skin microbiota and defense peptides, components of host innate immunity, interact on amphibian skin to provide protection from infection?
- Can a probiotic strategy be designed to successfully ameliorate the effects of chytridiomycosis?

The focus of these studies is on a highly susceptible Discoglossid host, the midwife toad, *Alytes obstetricans*.

A complex interplay of interactions between the etiological disease agent, *Batrachochytrium dendrobatidis* (*Bd*) and components of innate immunity are elucidated by sampling hosts in their natural habitat and testing the effects of these isolated immune components under controlled laboratory conditions and semi-natural mesocosms. An innate immunity profile of the host is first characterized and individual components screened *in vitro* to identify candidates with beneficial probiotic agents (Chapter 1). Beneficial traits of a probiotic for treating *Bd*-infected hosts include the ability to inhibit pathogen growth while simultaneously persisting in the presence of the host's defense components at the skin surface. How the selected probiotics influence the established microbial communities of naturally *Bd*-infected tadpoles was evaluated *in vivo* with bioaugmentative therapeutic treatments in semi-natural mesocosms (Chapter 2) to improve host survival outcomes post-metamorphosis (Chapter 3).

Chapter 1 provides a diversity profile for two components of the innate immune system of the skin surface: a mixture of bioactive skin peptides and the indigenous microbiota. Surveys of cultivable microbes and defense peptides for *A. obstetricans* hosts were sampled at three stages of development. Unique isolates of bacteria and fungi were cultured from egg clutches, tadpoles, and adults and identified by closest sequence match. Grouped into 97%-similarity operational taxonomic units (OTUs; a proxy for species-level identification), 60 unique bacterial and 23 unique fungal OTUs were identified. Expression of the alyteserins, a family of antimicrobial peptides, was present in adults and metamorphs and a subset of these peptides was found at the tadpole stage, whereas no peptides were

detected on the egg clutches. A "core" of shared membership of peptides, bacteria, and fungi was identified among developmental stages and between populations.

The isolation and identification of resident bacteria and natural peptide mixtures, along with a *Bd* isolate also cultured from a moribund *A. obstetricans*, provided the components with which to test microbe-host-pathogen interactions under controlled conditions. The ability of bacterial metabolites to inhibit the growth of *Bd* immune function of each bacteria was evaluated by a challenge assay. Similarly, *Bd* and bacterial growth was challenged by host skin peptides. Assays of crude skin peptide mixtures revealed host capacity to inhibit *Bd* and simultaneously inhibit or promote the growth for different symbiotic bacteria. While a majority of the symbiotic bacteria surveyed displayed *Bd*-inhibitory properties, metabolites from some isolates enhanced the growth of *Bd*. Antifungal metabolite production was common to certain bacterial families such as the Flavobacteriaceae, where all isolates consistently inhibited *Bd* growth. It is less clear within the Pseudomonadaceae family where mixed effects of inhibiting and enhancing pathogen growth were observed. A minimum of one *Bd*-inhibitory OTU isolate was detected in all adult individuals surveyed, perhaps contributing to the persistence of these uninfected toads in a *Bd*-positive region by herd immunity, whereby risk of disease outbreak in a community is minimized when a significant proportion of the community immunized, or protected, from the pathogen. These findings suggest that disruptions in the delicate balance between host-produced and host-acquired innate immune defenses may likely increase susceptibility to infection and disease.

With culture-independent microbial fingerprinting techniques, **Chapter 2** traces bacterial and fungal community structures in hosts through metamorphosis and after microbial manipulation. An experimental trial conducted with *A. obstetricans* tadpoles collected from four geographically distinct populations under semi-natural mesocosm conditions, shows that the initial community structure established for a host within a population is specific. This specificity is maintained through metamorphosis and after treatment with a probiotic therapy. That the microbiota differ among host species was only recently demonstrated and the results in this trial demonstrate that microbiota also differ among populations within a species. A core microbiota featuring shared microbial membership is described across development and among populations. The sampling for bacterial and fungal communities took place at three points in the trial: upon *A. obstetricans* collection from natural ponds, a week after treatment with a probiotic, and upon

metamorphosis. Structural community analyses were complemented with metagenomic analysis for a subset of samples taken from the second time point, after treatment. Among treated individuals, shifts in microbial community taxa and overall abundance were found in comparison to the controls. In addition, skin peptides and individual members of the microbial community sampled in metamorphs at the third timepoint in this trial were correlated. An indirect benefit is conferred when components of the skin exudates enhance the growth of specific strains of bacteria that in turn produce antifungals to inhibit pathogen colonization. Thus, a *pro*microbial (or prebiotic) role towards symbiotic microbiota may exist in contrast to the popularly-perceived *anti*microbial skin peptide mixtures. Thus I re-evaluate traditionally unidirectional roles of innate immunity factors in pathogen defense. Further rigorous experimental testing is warranted to disentangle potential symbioses that may have evolved between individual host peptides and indigenous microbial isolates.

The impact of the probiotic therapy administered at the tadpole stage on reducing *Bd* loads and improving survival outcomes post-metamorphosis is analyzed in **Chapter 3**. While several pilot studies have shown promise of probiotics to reduce infections and host morbidity under controlled laboratory conditions, this is the first study that examines the application of probiotics at the larval stage, under semi-natural conditions (a step preliminary to treatment in natural ponds). The tadpoles collected from four different populations harbored natural *Bd* infections and were assigned to two treatment groups: a probiotic therapy group and a control group that received only a sham treatment with sterile water but endured the same stress due to the capture process and duration of the sham treatment. The probiotic treatments featured *P. fluorescens* and *F. johnsoniae* bacteria isolated from adult toads in a population studied here. While infection in tadpoles were not significantly impacted by *P. fluorescens* a week after treatment, *F. johnsoniae* reduced loads in two populations compared to controls post-treatment. Upon metamorphosis, the interacting factors, population and ambient temperature, could also explain variation in *Bd* loads. Regardless of having no impact on *Bd* loads, survival for both probiotic-treated tadpoles (40% survival) was significantly improved compared to survival rates in control-treated tadpoles (25% survival). The suspected mechanism is that bacterial metabolites reduced *Bd* growth, as observed *in vitro.* However, this result is not consistent since infection loads varied, influenced by ambient temperature and season, returning lower loads at higher temperatures. The result of among-population variation in *Bd*-induced mortality supports previously conducted findings under controlled laboratory conditions. The absence of a significant treatment-by-population interaction may reflect low sample size, and, given

strong differentiation in initial microbial communities, future management strategies should proceed with care. Since benefits are not clearly conferred via direct inhibition of the pathogen's growth, further mechanistic studies are needed to understand how bioaugmentation is linked to improved survival.

ZUSAMMENFASSUNG

Die in dieser Dissertation vorgestellte Arbeit erforscht zeitgenössische Fragen der Krankheitsökologie im Zusammenhang mit einer drohenden Amphibienkrankheit, der Chytridiomykose: Wie interagieren Hautmikrobiota und Abwehrpeptide, Bestandteile der angeborenen Immunabwehr des amphibischen Wirtes, auf der Amphibienhaut um einen möglichst guten Schutz vor einer Infektion zu bieten? Ist es möglich, eine Strategie zu entwickeln, die mittels Probiotika die Auswirkungen von Chytridiomykose mildert? Der Schwerpunkt dieser Studien liegt auf einem sehr anfälligen Wirt, der Geburtshelferkröte *Alytes obstetricans*.

Das komplexe Zusammenspiel von Wechselwirkungen zwischen dem ursächlichen Erreger der Chytridiomykose, *Batrachochytrium dendrobatidis* (Bd), und den Komponenten der angeborenen Immunabwehr kann mit verschiedenen Methoden untersucht werden. Ein Ansatz besteht darin Wirtstiere in ihrer natürlichen Umgebung zu beproben und die aus den Proben isolierten Immunkomponenten unter kontrollierten Laborbedingungen sowie in naturnahen Mesokosmen auf ihre Auswirkungen hin zu testen. Zunächst wird das Profil der angeborenen Immunabwehr des Wirtes charakterisiert, das in einem zweiten Schritt *in vitro* auf Einzelkomponenten mit vorteilhaften probiotischen Eigenschaften hin untersucht wird (erstes Kapitel). Probiotische Mikroben, die zur Behandlung von infizierten Wirten eingesetzt werden könnten, haben die Fähigkeit, das Wachstum von Krankheitserregern zu hemmen und sind gleichzeitig resistent gegen die wirtseigenen Abwehrpeptide auf der Hautoberfläche des Wirtes *A. obstetricans*. Wie die ausgewählten Probiotika die etablierten mikrobiellen Gemeinschaften von natürlich mit *Bd* infizierten Kaulquappen beeinflussen, wurde *in vivo* mit einer bioaugmentativen therapeutischen Behandlung in naturnahen Mesokosmen (Kapitel 2) untersucht, um so das post-metamorphe Überleben des Wirtes (Kapitel 3) zu verbessern.

Das **erste Kapitel** stellt Diversitätsprofile zweier Komponenten des angeborenen Immunsystems vor, die auf der Hautoberfläche von Amphibien zu finden sind; diese Profile bestehen aus einer Mischung bioaktiver Hautpeptide und indigener Mikrobiota. Hierbei wurden drei unterschiedliche Entwicklungsstadien des Wirtes *A. obstetricans* auf kultivierbare Mikroben und Abwehrpeptide untersucht. Einzelisolate von Bakterien und Pilzen wurden aus Eigelege, von Kaulquappen und Adulten kultiviert und durch ein, closest sequence match' identifiziert. Alle Einzelisolate mit einer Ähnlichkeit von mindestens 97%

wurden in operative taxonomische Einheiten (OTUs; ein Proxy für Arten-Level-Identifikation) gruppiert. Dadurch konnte ich insgesamt 60 spezifische bakterielle und 23 spezifische Pilz- OTUs identifizieren. Bei den Hautpeptiden zeigten Adulte und Metamorphe eine Expression von Alyteserinen, einer Familie antimikrobieller Peptide, die teilweise auch auf Kaulquappen gefunden werden konnten; auf Eigelegen konnten keine Alyteserine nachgewiesen werden. Insgesamt konnte ein "Kern" an gemeinsamen Peptiden, Bakterien und Pilzen identifiziert werden, der auf verschiedenen Entwicklungsstadien und Populationen gleichzeitig vorkommt. Eine genauere Untersuchung der Mikroben-Wirt-Pathogen-Interaktion unter kontrollierten Bedingungen wurde durch zwei Faktoren ermöglicht: Einerseits durch die Isolierung und Identifizierung wirtsansässiger Bakterien und natürlicher Peptide, und andererseits durch die Identifizierung eines *Bd*-Isolates, das von einem moribunden *A. obstetricans* Individuum kultiviert wurde. In diesem Zusammenhang evaluierte ich mittels eines challenge assays die Fähigkeit bakterieller Metabolite das Wachstum von *Bd* zu inhibieren. Nach dem gleichen Prinzip untersuchte ich den Effekt von *A. obstetricans* - Hautpeptiden auf das Wachstum von *Bd* und der Mikrobiota. In den Assays wurden unaufbereitete Mischungen aus Hautpeptiden verwendet. Die Resultate legen nahe, dass das angeborene Immunsystem von *A. obstetricans* sowohl das Wachstum von *Bd* hemmt, als auch das Wachstum von unterschiedlichen symbiotischen Bakterienisolaten jeweils hemmen und fördern kann. Während die Mehrheit der untersuchten symbiotischen Bakterien *Bd*-inhibierende Eigenschaften hatte, fand ich trotzdem Metabolite einiger Bakterien, die das *Bd*-Wachstum förderten. Die Metabolite einiger Bakterienfamilien, besonders der Flavobacteriaceae, hatten hingegen konsistent eine antifungielle Wirkung, d.h. wachstumshemmende Eigenschaften in Bezug auf *Bd*. Die Familie der Pseudomonadaceae hingegen zeigte kein klares Muster, da sowohl wachstumshemmende, als auch wachstumsfördernde Eigenschaften festgestellt werden konnten. Alle untersuchten adulten *A. obstetricans* wiesen mindestens ein Isolat auf, das hemmend auf *Bd*-Wachstum wirkt. In diesem Zusammenhang könnte die sogenannte Herdenimmunität eine mögliche Erklärung für das Überleben der Populationen in einem *Bd*-positiven Habitat darstellen: Die Gefahr eines Krankheitsausbruchs in einer Population wird auf ein Minimum reduziert, wenn ein erheblicher Anteil der Population gegen den Erreger immunisiert oder geschützt ist. Meine Befunde legen nah, dass Störungen des empfindlichen Gleichgewichts zwischen der angeborenen und erworbenen Immunabwehr die Anfälligkeit für Infektionen und Krankheiten erhöhen.

Kapitel zwei beschreibt die Strukturen der Bakterien- und Pilzgemeinschaften, die auf metamorphisierenden, mit Mikroben behandelten Wirten angesiedelt sind. Diese Mikroben-Gemeinschaften wurden mittels der mikrobiellen Fingerprinting-Technik bestimmt, die unabhängig davon ist, ob Mikroben kultiviert werden können. Ich konnte durch eine experimentelle Mesokosmosstudie an *A. obstetricans* Kaulquappen aus vier geographisch getrennten Populationen zeigen, dass die Mikrobengemeinschaft eines Wirtes innerhalb einer Population spezifisch ist. Diese Spezifität bleibt auch noch nach der Metamorphose erhalten, und bleibt auch nach einer probiotischen Behandlung unverändert. Erst kürzlich konnte gezeigt werden, dass sich die Mikrobiota unterschiedlicher Wirtsarten voneinander unterscheiden. Die Resultate meines Experimentes zeigen auf, dass sich sogar die Mikrobiota verschiedener Populationen innerhalb einer Art unterscheiden können.

Eine dauerhafte und gemeinsame Kern-Mikrobioten-Gesellschaft existiert sowohl für einen Wirt über den Verlauf seiner Entwicklung wie auch für Individuen derselben Population. Die strukturelle Beprobung für Bakterien und Pilzgesellschaften fand dreimal während des Experimentes statt: Nach dem Fang von *A. obstetricans* aus den Weihern, eine Woche nach der probiotischen Behandlung, und zum Zeitpunkt der Metamorphose. Diese strukturellen Analysen der Mikrobiota wurden mit einer metagenomischen Analyse ergänzt. Für die metagenomische Analyse verwendete ich einen Teil der Proben aus der zweiten Beprobungsrunde (nach der probiotischen Behandlung). Bei den behandelten Individuen wurde festgestellt, dass sich im Vergleich zu den Individuen der Kontrollgruppe sowohl die Zusammensetzung der mikrobiellen Taxa verändert hatte, als auch deren Abundanz. Ausserdem korrelierten Hautpeptide und bestimmte Mikroben zum Zeitpunkt der Metamorphose. Hautpeptide können nebst dem direkten Nutzen, der darin besteht, dass sie das Wachstum von *Bd* direkt hemmen, einen indirekten Nutzen haben. Dieser entsteht, wenn die Hautsekrete das Wachstum von bestimmten Bakterienstämmen, die ihrerseits antifungielle Sekrete produzieren, begünstigen. Möglicherweise existiert also eine *pro*-mikrobielle Funktion von Hautsekreten, was im Kontrast zur gängigen These *anti*-mikrobieller Hautsekrete steht. Deshalb wäge ich die traditionell einseitig wahrgenommene Rolle der angeborenen Immunabwehr in der Bekämpfung von Pathogenen neu ab. Weitere rigorose experimentelle Tests sind nötig, um mögliche Symbiosen, die zwischen den Peptiden des Wirtes und mikrobiellen Isolaten bestehen könnten, nachzuweisen.

In **Kapitel 3** wird analysiert, wie eine probiotische Therapie während dem Kaulquappenstadium die Stärke der Infektion mit *Bd* und das Überleben nach der Metamorphose beeinflusst. Mehrere Pilotstudien konnten zeigen, dass probiotische

Wirkstoffe unter Laborbedingungen Infektionen und Krankheitsverläufe mildern können. Dies ist jedoch die erste Studie, die die Wirksamkeit von probiotischen Therapien während der Larvalphase und unter naturnahen Bedingungen testet (als Zwischenschritt zur Behandlung im Teich). Die Kaulquappen, die aus vier verschiedenen Populationen entnommen wurden und natürlicherweise bereits mit *Bd* infiziert waren, wurden in zwei Behandlungsgruppen unterteilt: zwei Gruppen erhielten je eine probiotische Therapie, während die dritte Gruppe zwar derselben Prozedur ausgesetzt war, jedoch nur mit sterilem Wasser "behandelt" wurde. Die probiotischen Therapien bestanden entweder aus *P. fluorescens* oder *F. johnsohniae* Bakterienisolaten. Diese wurden von adulten Kröten einer hiesigen Population isoliert. Während die Infektion der Kaulquappen durch *P. fluorescens* eine Woche nach der Behandlung unverändert war, reduzierte *F. johnsoniae* die Infektionsstärke im Vergleich mit der Kontrollgruppe bei zwei Populationen. Zum Zeitpunkt der Metamorphose liess sich auch ein Effekt der Herkunftspopulation und der Umgebungstemperatur auf die *Bd*-Infektionsstärke nachweisen. Obwohl die *Bd*-Infektionsstärke der Kaulquappen nicht durch die probiotische Behandlung beeinflusst wurde, war das Überleben beider behandelten Gruppen (40%) signifikant höher als das der Kontrollgruppe (25%). Der Mechanismus dahinter ist vermutlich eine Reduktion des Pilzwachstums durch Bakterienmetabolite, wie dies auch *in vitro* beobachtet wurde. Da die Infektionsstärke aufgrund der Umgebungstemperatur und der Saison variierte, sodass tiefere Infektionsstärken bei höheren Temperaturen beobachtet wurden, ist dieser Befund jedoch nicht konsistent. Wie bereits in früheren Studien unter kontrollierten Laborbedingungen gezeigt wurde, variierte auch in meinem Experiment die Mortalität durch *Bd* in verschiedenen Populationen. Das Fehlen einer signifikanten Interaktion von "Behandlung" mit "Population" könnte an einer zu geringen Stichprobengrösse liegen. Da die indigenen Mikrobiota der Population sich stark unterscheiden, sollten zukünftige probiotische Managementstrategien mit Vorsicht entwickelt werden. Der Nutzen einer probiotischen Behandlung konnte nicht eindeutig anhand einer direkten Inhibition des Pilzwachstums nachgewiesen werden. Daher sind weitere mechanistische Studien nötig um zu verstehen, wie probiotische Behandlungsansätze das Überleben verbessern können.

INTRODUCTION

Amphibians in crisis

A third of approximately 7,000 amphibian species are currently threatened with extinction (Stuart, 2008; Stuart *et al.*, 2004). Since scientists first shared anecdotal concerns of alarming species disappearances during the 1989 World Congress of Herpetology, evidence for declines at rates unprecedented over geological time has mounted (Blaustein *et al.*, 2013; Lips *et al.*, 2013). This has summoned a response to simultaneously understand, and more recently, to actively reverse these declines (Gascon et al., 2005; Woodhams et al., 2011). Several anthropogenic causes have been cited: habitat loss, climate change, UV-B susceptibility, overexploitation, introduced species, the pet trade, as well as a complex interaction of these factors (Kiesecker *et al.*, 2001). A majority of the underlying declines are enigmatic, occurring in pristine areas purportedly removed from direct anthropogenic reach (Crump *et al.*, 1992; La Marca *et al.*, 2005; Laurance *et al.*, 1996; Stuart *et al.*, 2004), though not beyond the reach of an infectious pathogen.

The study host

One species threatened in central Europe is the terrestrial midwife toad, *Alytes obstetricans* (Discoglossidae; Linnaeus, 1768). In Switzerland, where the species is currently listed as "endangered" by the ICUN Species Red List, nearly half of the toads have cryptically declined since the 1980's (Schmidt & Zumbach, 2005). Adult males in this species exhibit parental care by transporting fertilized egg clutches strung around their hind legs until the larva are deposited at a pond where tadpoles may forgo metamorphosis until the following Spring (Meyer, 2009; Thiesmeier, 1992).

Bd: an emerging pathogen

During hibernation, tadpoles in this species are susceptible to heavy oral infections of the contagious fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). The only pathogenic member of the Chytridiomycota (Pessier *et al.*, 1999), zoosporangia release motile zoospores that attack keratinized cells (Longcore *et al.*, 1999). Since keratin sheaths are limited to the mouthparts at the at tadpole stage, infections are localized. During metamorphosis, however, as the host undergoes reorganization of the organs and immune system (Rollins-Smith, 1998), *Bd* may spread to the newly keratinized cells of the body surface (particularly the forelimb and hindlimb digits and the drinking patch). With an

increased surface area available for pathogen colonization, is also an increase in infection burdens (Vredenburg *et al.*, 2011), inducing hyperkeratinosis (Pessier *et al.*, 1999). At this time, infection with zoospores could escalate to a lethal threshold (Vredenburg *et al.*, 2011) which, coupled with immune suppression during metamorphosis (Rollins-Smith *et al.*, 1997) may lead to *Bd*-induced chytridiomycosis mortalities. Fatality within days post-metamorphosis is common in various species (Carey *et al.*, 2006). Up to 90% mortality has been observed in a population of *A. obstetricans* (Tobler & Schmidt, 2010). Immediate lethal effects associated with the larval stage, prior to undergoing metamorphosis, have rarely been observed for *A. obstetricans* (though not for other species, such as *Bufo bufo),* nonetheless, direct, sub-lethal costs of harboring infections, can affect survival probability of new metamorphs (Garner *et al.*, 2009).

Although chytridiomycosis is an amphibian-specific disease (Skerratt *et al.*, 2004) and the proximate cause for amphibian declines (Stuart *et al.*, 2004), there is much heterogeneity within the pathogen-host system. One reason *Bd* is considered an "emerging" disease is on the basis of what was originally thought to be an evolutionarily conserved genome (Rosenblum *et al.*, 2013). However, variation among *Bd* strains is linked to different virulences and mortality rates through host infection experiments (Farrer *et al.*, 2011; Fisher *et al.*, 2009; Retallick & Miera, 2007). Disease outcomes are varied and depend on many interacting factors including: temperature, behavior, population, species, infection burden, density dynamics, and among-population differences in susceptible species (Bosch *et al.*, 2013; Savage & Zamudio, 2011; Tobler & Schmidt, 2010; Venesky *et al.*, 2011). Within a susceptible species, some populations may still persist despite pathogen presence (Briggs *et al.*, 2005). In *A. obstetricans,* mass mortalities linked to chytridiomycosis have been documented in Spain (Bosch *et al.*, 2001). However, individuals at these sites report comparable infection loads with samples of hosts in Switzerland, where a direct impact on populations is less visible and dramatic (Garner *et al.*, 2009). While there is experimental evidence of chytridiomycosis-induced mortalities within *A. obstetricans* populations in Switzerland (Tobler & Schmidt, 2010), curiously, no detrimental impact of *Bd* is linked to population growth rates across the country (Tobler *et al.*, 2012).

Conservation strategies

Scientists are mobilizing conservation plans to tackle what has been described as the most devastating disease known to a vertebrate taxon (Gascon et al., 2005). Effective conservation strategies rely on controlling *Bd*. Several methods to manage *Bd* have been proposed: (1) preventing spread to naïve populations, (2) captive breeding programs for

susceptible species, and (3) mitigation of *Bd* in the wild. Mitigation methods are being tested but there is currently no prescribed method for threatened amphibians in the wild (Woodhams *et al.*, 2012b). One strategy is to treat host infections with chemical agents (Geiger & Schmidt, 2013), another is to indirectly reduce *Bd* prevalence through ecological bio-control methods (Buck *et al.*, 2011), and a third, promising possibility given an indirect intervention, long-term effects, and ecologically sound means, is to enhance immunity to benefit host health (Harris *et al.*, 2009a; Vredenburg *et al.*, 2011; Woodhams *et al.*, 2011).

Amphibian immunity

Amphibian immunity relies on both innate and adaptive immune mechanisms. An individual's immunocompetence, or disease resistance, will depend on these components (Savage & Zamudio, 2011; Woodhams *et al.*, 2007a). Disruption or deficiency in either component will reduce immunocompetence (Ramsey *et al.*, 2010).

The skin can be thought of as an ecosystem (Fredricks, 2001; Rosenthal *et al.*, 2011); stability within the ecosystem can affect host health and survival. As an organ, the skin fulfills basic physiological functions including osmoregulation, solute transport, and, cutaneous respiration. An interface separating the host from its environment, the skin is also a physical barrier, protecting the host from pathogens while harboring microbes that may influence host health or induce disease. Host defense from zoospore attack is first determined upon contact with the first line of defense, the skin surface. In order to encyst (Pessier *et al.*, 1999), the zoospore must evade or compete with a multitude of other microorganisms, lysozyme, defense peptides, and bacteriocins produced by the symbiotic bacteria present at the mucosal layer.

Antimicrobial skin peptides

The skin peptide repertoire of amphibians varies with each species in terms of number and structural diversity of endogenously synthesized peptides and is widely studied as a mediator of innate immunity (Conlon, 2011a; Hancock & Diamond, 2000; Nizet *et al.*, 2001; Simmaco *et al.*, 1998; Zasloff, 2002). Crude skin mixtures and isolated peptides display a broad spectrum of antimicrobial potencies that include inhibition of *Bd* growth at both constitutive (Pask et al., 2012) and induced concentrations (Rollins-Smith et al., 2002b). The efficiency of pathogen inhibition *in vitro* varies with species (Rollins-Smith *et al.*, 2002a; Rollins-Smith *et al.*, 2006) and expression is regulated by pathogen infection loads (Woodhams *et al.*, 2012a). Several microbial pathogens may inhibit AMP potency (Schadich & Cole, 2009) and in turn, symbiotic microbes may induce host peptide synthesis

(Simmaco *et al.*, 1998). Various studies have concluded antimicrobial activity of amphibian peptides (Uccelletti *et al.*, 2010; Zasloff, 2006), including those of *A. obstetricans*, (Conlon *et al.*, 2009; Conlon *et al.*, 2012) against clinically-relevant human pathogens. Fewer studies have examined interactions of AMPs with microbes present in the natural habitat of the amphibian skin surface (Rollins-Smith *et al.*, 2002b). However as not all amphibian taxa synthesize and express AMPs, it is hypothesized that such species lacking a peptide repertoire may compensate by hosting an array of bacteria that accomplish host defense tasks (Conlon, 2011b).

Skin microbiota and disease management

Host-acquired microbial symbionts are also found together with the rich array of host-produced AMPs at the mucosal surface. There are hundreds of microorganisms residing in communities on the skin surface of vertebrate hosts including bacteria, fungi, and protists which an invading pathogen must evade or compete with to reach the host (Dominguez-Bello *et al.*, 2010; Fredricks, 2001). Microbes on the skin surface have long been positioned within the narrow spectrum between pathogen and innocuous commensal (Cogen *et al.*, 2008).

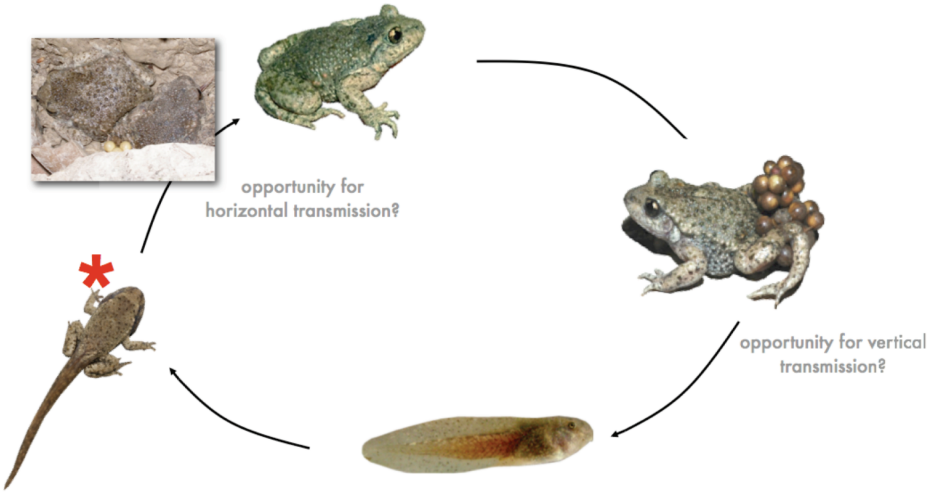

Figure 1. Schematic illustrating life-history cycle of the host, the midwife toad*, A. obstetricans,* and various life-stages for opportunities of vertical and horizontal transmission of innate immune defense or probiotics. The * designates the critical stage post-metamorphosis targeted to prevent Bd –associated mass mortalities.

Collaborations between microbiologists and immunologists have led to conceptual revisions of host-pathogen and host-symbiont interactions, in recognition of beneficial role of

residential microbes that influence host health in the residential microbiota (Cogen *et al.*, 2008; Dethlefsen *et al.*, 2006; Rosenthal *et al.*, 2011). The field of eco-immunology additionally factors-in environmental interactions encountered by the host's immune system (Rollins-Smith & Woodhams, 2012), whereby the skin surface is a model for studying the interface of host-produced and host-acquired components that together comprise an integrated innate immunity.

The diversity of the skin microbiota has been profiled for several amphibian species, revealing a prevalence of symbiotic bacteria with antifungal metabolites that could strategically be applied to potentially influence host health (Flechas *et al.*, 2012; Lauer *et al.*, 2008; McKenzie *et al.*, 2011; Woodhams *et al.*, 2007b). Various factors influence the composition of the microbiota including genotype, microbial interactions, and a genetic component (Dethlefsen *et al.*, 2006). Composition and structure of the microbiota can predict disease dynamics (Belden & Harris, 2007). Understanding the contribution of the taxonomic and functional diversity of the skin microbiota with natural interactions between the host and pathogen would contribute to better selection of beneficial microbes for disease management (Bletz *et al.*, 2013; Cogen *et al.*, 2008; Hancock *et al.*, 2012).

A probiotic is defined as a viable culture of microorganisms that beneficially influence host health (Schrezenmeir, 2001). The probiotic should positively interact with host-produced defenses such as AMPs, while maintaining defensive properties for *Bd* inhibition (Bletz *et al.*, 2013). Various candidates have been identified and show promise in controlled trials (Harris *et al.*, 2009b). One such candidate, *Janthinobacter lividum,* produces the antifungal metabolite violacein and has been isolated from various terrestrial and semi-aquatic amphibian hosts, a positive trait for a candidate probiotic (Bletz *et al.*, 2013).

Host health in a susceptible species could be managed strategically through probiotic therapeutic treatments that target a critical stage within the biphasic life cycle of an amphibian (Fig. 1), an important consideration in the long-term survival of a host. Likewise, as most *Bd*-associated mortalities of *A. obstetricans* in Switzerland have been observed to occur shortly post-metamorphosis, probiotics that attenuate infection loads before the culmination of metamorphosis, may improve chances of host survival long-term. While little is known as far as the heritability of microbiota in amphibians, there is potential for social transmission of beneficial microbes, as shown by Walke *et al.,* (2011) in parent-to-offspring microbe transfer in glass frogs. The cross-generational, long-term sustainability component that is possible with microbial therapies is one aspect that favors probiotic treatments over the one-time impact of chemical agents in *Bd* disease mitigation (Woodhams *et al.*, 2011).

What to expect in this dissertation

Much groundwork on innate immune defenses in amphibians has been done (Brucker *et al.*, 2008; Harris *et al.*, 2009a; Harris *et al.*, 2006; Lauer *et al.*, 2007; Woodhams *et al.*, 2007b), yet fundamental questions in disease ecology still remained when work for this PhD began in 2008. Little was known about factors that discriminate microbial community makeup. Is the microbiota species-specific? If so, as revealed in 2011 by McKenzie *et al.*, is this associated with the disease susceptibility of a species? Do populations within a species vary and can this explain population-level variation of *Bd*-associated mortalities? Is microbial community structure stable across metamorphosis? How does an established community respond to microbial disturbance? Within the ecosystem of the host, what are the individual immune component roles and are they context-dependent? Is there promise for probiotic therapies in natural ponds of susceptible hosts?

Ultimately, the main aim of this PhD was to identify constituents within the integrated amphibian innate immune system that could be isolated and augmented to enhance host health to mitigate disease outcomes for a susceptible species. *In vitro* testing that simulates interactions of individual innate immunity constituents have unveiled a role of host-directed defense, wherein residential microbes are beneficial and antimicrobial peptides may promote the persistence of the beneficial members of the microbiota.

The chapters that follow comprise an integrative approach, incorporating methods from biochemistry, microbiology, and immunology to address microbial ecology questions. Chytridiomycosis drives the eco-immunological framework for elucidating pathogen-host-defense interaction dynamics. The role of innate immune components - skin peptides, and by extension, the symbiotic microbiota in *A. obstetricans* are described (Chapter 1), isolated interactions tested under controlled and mesocosm conditions (Chapter 2) and selected probiotics evaluated as a bioaugmentation therapy of infected hosts in a semi-natural pond experiment (Chapter 3).

Evaluating components of host-expressed and host-acquired constituents of the innate immune interactions through experimental-based testing further support a conceptual re-evaluation of formerly described roles of host defense. With this thesis, I propose to extend the view to a bidirectional amphibian innate immune defense with a host-directed symbiosis with select symbiotic microbes.

Note: Chapters are presented as independent manuscripts, with the second chapter submitted for publication. Thus, some material and methodologies are inevitably redundant.

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CHAPTER ONE

Towards the *in vitro* selection of a probiotic: a survey of the natural skin symbionts and peptide defenses harbored by the midwife toad across three life stages and their interactions with a fungal pathogen

Leyla R. Davis and Douglas C. Woodhams

ABSTRACT

Immunological ecology has not historically incorporated host microbiota. However, recent technological advances in microbial ecology have drawn attention to the vast microbiome harbored by all organisms that strongly influence host functioning, extend host phenotype, and impact host health. Thus, an ecological study of emerging wildlife diseases such as chytridiomycosis, which impacts thousands of amphibian species, can benefit by merging microbiota into an immunological framework. In this study, we sampled the microbiota and skin peptides from the midwife toad, *Alytes obstetricans,* which, along with related *Alytes* species has experienced mass mortalities and population declines and can be highly susceptible to infection by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) as tadpoles and subsequently experience high mortality upon metamorphosis. We detected 100% *Bd* infection prevalence in tadpoles from two populations in Switzerland, but no infection in adults. We isolated 299 bacteria and 96 fungi from eggs (n=10), tadpoles (n=16), and adults (n=19), and also collected surface peptides potentially used for pathogen defense. Isolates were identified by sequencing 16S rRNA and ITS1 genes and grouped into 97% similar, species-level operational taxonomic units (OTUs; 60 bacteria, 23 fungi). Skin peptides were capable of inhibiting *Bd*, and could also either inhibit or enhance skin microbes. Many of the bacterial symbionts could inhibit *Bd* in co-culture assays, although filtrate from some bacteria also enhanced *Bd* growth. A minimum of one *Bd* inhibitory isolate was detected in all but two (90%) of adult individuals surveyed, perhaps contributing to their persistence in a *Bd*-endemic region and lack of infection as adults. By testing the interactions among fungal pathogen, host defense peptides, and microbiota, we are positioned to select potential probiotics and predict the effects of skin peptides as prebiotics. Candidate probiotics extend the host innate immune system by inhibiting *Bd* growth and have the capacity to withstand host defense peptides which may assist in

establishment and persistence within the skin microbial community. Incorporation of the microbiota into immunological ecology will make strides toward medical and conservation applications.

INTRODUCTION

As the largest vertebrate organ, the skin provides a physical barrier between host and environment. At this interface, the skin ecosystem is the first line of defense with complex interactions between microbial pathogens and symbionts, and host mucosal immune defenses that include antimicrobial peptides (AMPs) (Fig. 1). The role of organisms that inhabit the skin surface has recently undergone a shift in perception due, in part, to a burgeoning of studies on the subject (Dominguez-Bello *et al.*, 2010; Rosenthal *et al.*, 2011; Schmitt *et al.*, 2012). Microbiota are becoming accepted components of healthy hosts that influence disease susceptibility in humans (Fredricks, 2001; Rosenthal *et al.*, 2011) and more recently, in wildlife (Daskin & Alford, 2012). Host microbiota (including bacteria and fungi) provide nutrients, exclude pathogens, and develop immune function. How we view microbes in recent years has been influenced by an influx of metagenomics and next-generation sequencing. Consequently, recent ecological research aims to examine variability in microbial communities whose membership may be dependent on host species, population of origin, anatomy, habitat, time of year, and developmental stage, among other factors (Costello et al., 2009).

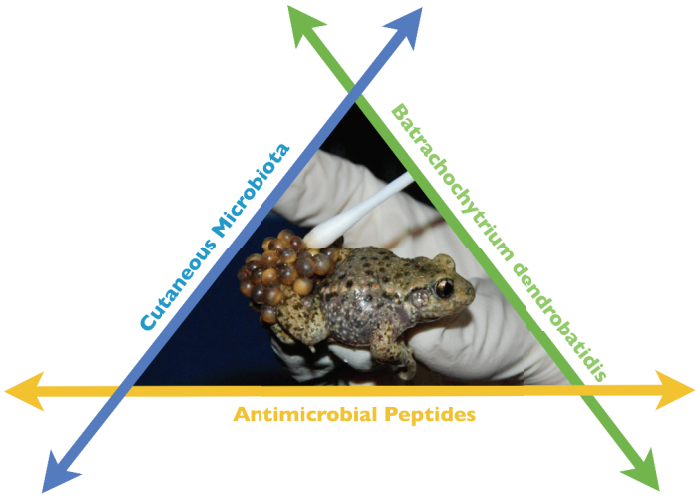

Figure 1. Model for interactions at the skin surface investigated in this study between host defense, the cutaneous microbiota, and the fungal pathogen, *Bd*. Microbes were screened to meet *in vitro* probiotic criterion at each node of interaction: ability to inhibit pathogen (*Bd*) growth and ability to grow, uninhibited by the host peptides.

Uncovering this frontier of surface innate immune defenses is especially timely for the Amphibia. Faced with massive global population declines associated with chytridiomycosis, a fungal disease caused by the pathogen, *Batrachochytrium dendrobatidis* (*Bd*), one third of all species are currently threatened with extinction, more so than any other vertebrate taxa (Stuart *et al.*, 2004). The unique skin physiology of amphibians which permits both gas and nutrient exchange across the epithelium, and their aquatic-terrestrial

biphasic lifecycle exposes amphibians vulnerable hosts to this infectious skin pathogen. Here, the symbiotic microbiota include fungal or bacterial colonists indigenous to the amphibian skin's epithelial surface. These may fulfill several roles on the continuum from mutualists (in symbiosis to help the host), commensals (non-disease causing residents), and opportunistic pathogens, though normally non-disease causing (Woodhams *et al.*, 2007b). Culturing microbes is a first step towards characterizing the existing natural microbiota. Cultures are needed to perform *in vitro* tests of interactions with the host's innate immune defenses and with potential pathogens. Improved understanding of such interactions would contribute to the development of management applications including probiotic treatments against disease.

Despite common unique features in skin physiology, there is extensive variation at the microbial and peptide composition levels within the diverse order Amphibia that extends across some nearly 7,000 species. A better understanding of this diversity would better inform future mitigation strategies that aim to provide prophylactics at the skin barrier of specific species (Woodhams *et al.*, 2011). However, there is a paucity of research on amphibian MCs to determine whether MC variability is species, site, temporal, or developmental stage-specific (McKenzie *et al.*, 2011). Amphibian skin-associated bacteria are shown to affect host susceptibility of *Bd*. Specific bacterial isolates have been shown to inhibit *Bd in vitro*, and successfully been applied as probiotics to reduce morbidity in diseased individuals (Brucker *et al.*, 2008).

A candidate probiotic would fulfill some of the prospective criterion (Bletz *et al.*, 2013; Klaenhammer & Kullen, 1999):

1. Inhibits growth of the *Bd* pathogen. This may be through direct (via metabolic toxins) or indirect (competitive exclusion, stimulation of other beneficial bacteria with antifungal compounds) tactics.
2. Persists in the presence of ambient host and granular skin peptide expression.
3. Is a symbiotic resident of the amphibian's microbiota; specifically to the species and population-of-origin in order to increase the likelihood of colonizing the target host.

The aim of this work is to describe the microbiota – the symbiotic bacteria and fungi that persist after rinsing and after expression of host antimicrobial peptides – and simultaneously screen for candidate probiotics. A comparison of innate immunity (peptides and microbes) is profiled at various stages of development.

METHODS

Field site and study species

Adult *A. obstetricans* toads (n=19) were collected from two populations in Kanton Basel, Switzerland on 27 May 2009 (Table S1). Twelve adults were found beneath rock crevices and dried leaf litter along a hillside above the Itingen breeding pond (47° 27′ 38″ N, 7° 47′ 02″ E, 410 masl) and each of the seven Zunzgen adults were collected from the muddy or rocky crevices offered by a turned-over tree trunk above the breeding pond (47° 26′ 5.30″ N, 7° 47′ 54.55″ E, 483 masl). Ten males (six from Itingen and four from Zunzgen) carried egg clutches on their hindlimbs, and both the clutch and adult were sampled. Each individual was handled with single-use rinsed vinyl gloves and coaxed into an autoclaved plastic tub for temporary housing prior to sampling for peptides, resident bacteria, fungi and detection of *Bd* before being returned to the site of collection the same night.

In a separate survey, tadpoles (n=16) were collected from the ponds one week later for capturing culturable bacteria present at the tadpole stage. To characterize host peptides across life stages, 12 adult *A.obstetricans* were sampled in August, 2008 as reported in Conlon *et al.* (2009). After breeding these toads in captivity, 25 tadpoles and 5 metamorphs were sampled for peptides and pathogen inhibition *in vitro.*

Sampling adults, tadpoles, and egg clutches for peptides and microbes

To collect peptides, granular gland secretions were induced by administration of norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri) by submersion in a bath (100 μM; tadpoles) or by subcutaneous injection (40 nmoles per g body mass of adult or metamorph; (Rollins-Smith *et al.*, 2002; Woodhams *et al.*, 2006). Eggs were sampled by swabbing, preserved in 90% methanol. Peptides were stored at -22 ° C according to protocol (Conlon *et al.*, 2009). To avoid undue stress on adults carrying egg clutches (n=8), ambient peptides were collected without norepinephrine stimulation by swabbing the adult dorsal skin and the egg clutch with separate cotton swabs for approximately 30 sec and stored in 90% methanol.

To sample symbiotic microbiota, the skin surface was briefly rinsed with MiliQ sterile water in order to remove debris and limit sampling of transient bacteria (Lauer *et al.*, 2007) prior to methodical sampling of the microbiota. The entire skin surface, beginning with each of the digits of the hindlimbs and forelimbs, followed by the flanks, ventral, and dorsal surfaces were swabbed (10x each). This procedure was repeated with three separate

Rayon-tipped wands (no. 155c, COPAN Italia, Bescia). One person swabbed all individuals to reduce any bias in swabbing technique (pressure applied, stroke style) and for a consistent sampling of the microbiota. The first swab was stored at -22°C for *Bd* diagnostics. In effort to capture as much of the spectrum of culturable bacterial and fungal microbiota, two types of culture media were used: a low nutrient 2% RIIA agar plate (RIIA; BD Difco™, Allschwil Switzerland) and a sabouraud dextrose media (SAB; Oxod AG, Switzerland) plate, each inoculated with separate swabs. The swab tip was streaked to the media surface in a zigzag pattern, rotating the wand to transfer all sampled microorganisms. Control plates inoculated with MiliQ rinse water were collected during the sampling process to capture any potential contamination. Plates were sealed with parafilm and incubated at room temperature (23**°**C).

In the separate survey of tadpole culturable bactera, the same sampling protocol was followed with the exceptions that: (1) no peptides were induced prior to sampling the culturable microbiota and *Bd*, and (2) in addition to the skin surface, buccal keratinized parts (where *Bd* infections are concentrated at the tadpole stage) were swabbed and transferred directly to RIIA agar plates. No swabs for culturable fungi were taken.

Real-time quantitative PCR diagnostic of *Bd* infection

To determine infection status and prevalence of *Bd*, DNA was extracted from swabs and analyzed by real time quantitative PCR according to Boyles *et al.* (2004) with a slight modification of the procedure to run 50 cycles. A FastStart Universal Probe Master was acquired from Roche, Chytridprobe, 6-FAM-CGA GTC GAA CAA AAT- MGB (Roche, Basel, Switzerland) and *Bd*-specific primers from Microsynth: ITS1 (5'-CCT TGA TAT AAT ACA GTG TGC CAT ATC TC-3') and 5.8s (5'-AGC CAA GAG ATC CGT TGT CAA A-3'). All runs were quantified based on zoospore standards acquired from EcoGenics (Zürich, Switzerland, 2008) and stored at -80°C.

Microbial culture and isolation

Prior to isolating unique bacterial and fungal colonies from the plates, RIIA plates were incubated at room temperature for 72 h, and SAB plates were incubated at room temperature for 7 d. Using sterile technique under a laminar-flow hood, unique morphotype colonies were selected, described in color, topology, gloss, border, and texture, and streaked onto plates for several iterations until a pure isolate was obtained. All bacterial and fungal isolates were given a unique ID linked to the host specimen, the original colony on the first growth plate, and number of subsequent iterations needed to obtain an axenic

isolate. A library of unique morphotypes (n=276) were cryogenically preserved and stored at -80°C for future work.

Sequencing bacterial and fungal isolates

Nucleotide sequences were obtained by Sanger sequencing with purified genomic DNA using universal primer pairs to obtain the broadest ecologically diverse sequence matches of both fungal and bacterial isolates. The DNA from each isolate was extracted from freshly inoculated, 72-hour old bacteria cultures (growing on RIIA or SAB plates) or 7-day old fungi (growing on SAB plates) with the Microbial Ultra Clean DNA Kit (MO BIO) designed for maximizing detection of fungi with PCR in soil (Karakousis *et al.*, 2006). The extracted DNA was then purified with the GenElute PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol until step four, modified to elute with 33 µl of the elution solution in order to concentrate genetic material.

16S rRNA genes were amplified with universal bacterial primer pairs: 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3') (Applied Biosystems) (Weisburg *et al.*, 1991) for obtaining the near-complete length of the hypervariable region for this gene (Frank *et al.*, 2008). Fungal isolates were amplified with primer pair, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') common in fungal community ecology studies (Horton & Bruns, 2001; Peay *et al.*, 2013). A 20 µL reaction was prepared with 10 µL Master Mix 2x, 1 µL of each primer (20 µM), 1 µL of bacterial or fungal genomic DNA, and 7 µL of nuclease-free water. Standard thermocycling conditions for 16s PCR products were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of a 30-sec 94°C denaturation, 1.5 min annealing at 55°C, a 2 min elongation at 72°C, and a final extension of 10 min at 72°C. The same thermocycling conditions were applied for fungi with a few modifications to the program: denaturation at 95°C, annealing at 54°C, and elongation was limited to only one minute. PCR products were separated on a 1% agarose gel and if a sample failed to amplify, replicates were performed and template concentration increased in the initial reaction. Negative control PCR reactions were performed with each set of amplifications and isolate extractions. PCR products were stored at -20°C for Sanger sequencing. DNA concentration of amplified DNA was determined by gel electrophoresis bands and quantified under a (NanoDrop) spectrophotometer (absorption at 260 nm). Samples were diluted with MilliQ water to a DNA concentration of 50 ng μl^{-1} and aliquots of the PCR product (200 – 500 ng

DNA) added to 1 µl of the respective forward primer for an 8 µl reaction. The forward sequences obtained by an ABI 3730 DNA Analyzer (Applied Biosystems). Raw sequences were edited for ambiguous Ns with the software PrimerDesign (v 2.1) and imported sequences were trimmed in Geneious (v 5.3) before obtaining a closest sequence match in the NCBI nucleotide database.

Phylogenetic analysis

Both 16S rRNA and ITS1 gene sequences were processed using the QIIME v6.0 pipeline (http://www.qiime.org). All viable sequences for isolates of bacteria (n=177) and fungi (n=97) were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold with a nearest neighbor algorithm (Hamady & Knight, 2009). This resulted in 60 bacterial and 23 fungal OTUs (described below). A representative set of sequences were then chosen by using the longest sequence for each OTU. Sequences were aligned to the Greengenes reference alignment (October 2012 version; greengenes.secondgenome.com) using PyNAST (Caporaso *et al.*, 2011) and a phylogenetic tree was constructed with RAxML v730 (Izquierdo-Carrasco *et al.*). All sequences were identified according to NCBI BLAST closest match. To better visualize phylogenetic relationships of the collective *A. obstetricans* bacterial community, a phylogenetic tree of bacterial isolates was visualized in Topiary Explorer software v6.0 (Pirrung *et al.*, 2011) and each branch corresponding to one of 60 OTUs was colored by four categories for affect on *Bd* growth: inhibitory, non-inhibitory, not significant, or a mixture of any of the previous outcomes as described in detail in the supplement section. All amplicon sequences were deposited (19 May 2013) in the EMBL-EBI database (http://www.ebi.ac.uk/) and accession numbers are pending. Taxonomies were grouped at the phylum, class, family, and OTU (or species) levels.

OTU diversity dataset

The final list of OTUs described above was prepared by removing replicates (see Table SX1) of an OTU isolated from the same individual. Our aim was to cover diversity of the microbiota and not abundance, given our isolation technique. OTUs overlapping with the control SAB plates that showed contaminant fungal growth were removed from the final list since these were not uniquely host-associated. With the final OTU list, richness was calculated per individual and compared among individuals, between populations, and between egg clutches and adults.

Mass spectrometry analysis

Crude skin secretions collected from adults were partially purified by passing over C-18 Sep-Pak cartridges (Waters Corp., Milford, Massachusetts), eluted in buffer containing 70% acetonitrile and 0.1% TFA, and spun dry with heat (Conlon *et al.*, 2009). Dry weight of peptide-enriched samples was measured before analysis by matrix-assisted laser desorption/ionization (MALDI) using an Autoflex I time-of-flight mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a 337nm nitrogen laser. A 20 µl sample solution at a concentration of 1 mg*ml^{-1} peptides was diluted with 20 µl H_2O + 0.1% trifluoroacetic acid, vortexed and 1 µl was spotted on a "Prespotted AnchorChip" target prepared with α-cyano-4-hydroxycinnamic acid as matrix (HCCA, Bruker). Instrument calibration was obtained using signals from the HCCA matrix at m/z 379.09 and a mixture of standard peptides composed of Bradykinin 1-7 (m/z 757.40), angiotensin II (m/z 1046.54), angiotensin I (m/z 1296.69), renin substrate (m/z 1758.93), ACTH clip 18-39 (m/z 2465.20) and Somatostatin 28 (m/z 3147.47) all obtained from the peptide calibration standard mix II (Bruker). An average of 300 manual shots was obtained per spectra, acquired within the molecular mass range of 900-3,000 m/z. Peptide profiles for all individuals were individually annotated for and exported for relative intensity of each peak. Molecular masses of peptides previously described were compared among life stages and all shared peptides calculated to test for differences in peptide profiles among individuals, between populations, and among life-history stages.

Assays challenging growth of *Bd* and cultured host bacteria

The following assays (summarized in Table S2) tested whether: (1) peptides could inhibit *Bd* growth, (2) bacterial filtrate could inhibit *Bd* growth, and (3) peptides could influence bacterial growth.

We tested interaction dynamics at each intersection of the host-defense-pathogen triangle (Fig 1.) under controlled *in vitro* conditions in 96-well flat-bottom microplates. To prepare for the assays, Swiss *Bd* (isolate no. 0739), originally isolated from a moribund adult *A. obstetricans* (Gamlikon, CH) was cultured for all the pathogen challenge assays. We chose this strain for its pathogencity (recovered from chytrid-host post-mortem) and to best simulate the natural interactions between pathogen strain and host bacteria metabolites present on the host's skin. We scraped the sides of a *Bd* culture flask to obtain active zoospores and poured 1 mL onto RIIA agar plates containing 1% tryptone (Sigma Aldrich, CH), tilting the plate to cover the entire surface. After air-drying (~10 min), plates were covered and sealed with Parafilm M® and incubated at 19° C for four days or until active

zoospores were observed under a microsocope. Motile zoospores were harvested by flooding each plate with 5 ml of t-broth, containing 1% Tryptone and .1% PenStrep (both acquired from: Sigma-Aldrich, St. Gallen, CH) for 20 min to stimulate zoosporangia and release active zoospores by lightly tilting the plate to capture zoospores in a solution collected by pipette and transferred to a sterile tube of stock. Concentration of zoospores was counted with a haemocytometer and the final stock solution diluted to a concentration of 10^5 zoospores per mL, and kept cool by an ice bucket for all challenge assays set-up on a given day.

To set-up a plate, each challenge well contained 50 μL of motile *Bd* zoospores in 1% tryptone media and 50 μL of filtered bacterial metabolites (or host peptides) in water, replicated five times. A negative-control consisted of heat-killed zoospores, 10 min. at 60° C, or heat-killed bacteria), a positive control column (live zoospores or bacteria) and up to five columns of the subsequent challenge assay(s). A border of T-broth-only wells provided a protective barrier from evaporation and external contamination. Plates were firmly sealed with parafilm (M® - Pechiney Plastic Packaging) and incubate under controlled temperature (19° C) and light conditions (15-hour photoperiod).

Growth was determined by absorbance readings obtained with a spectrophotometer at 492 nm. Plates were read at two time points: immediately following assay preparation (day 0, the baseline) and after seven days, a time period considered optimal for maximal *Bd* growth (Woodhams *et al.*, 2008). If any contaminant fungal or bacterial growth was observed, the plate was discarded and the assay redone. We standardized the assay protocol in order to develop a comparative method for each type of *in vitro* growth and to account for a potential block effect from plate to plate (see supplement for block study trial).

(1) Do host peptides inhibit Bd *growth?*

Natural mixtures of partially-purified skin peptides from metamorph *A. obstetricans* were challenged at a range of different concentrations between 25-500 μg/mL to active *Bd* zoospores to determine the minimal inhibitory concentration (MIC), or the lowest concentration at which no *Bd* growth occurred.

No significant population-level differences were detected in the peptide composition among individuals from the two populations, thus this representative *A. obstetricans*

metamorph's skin peptide mixture was used in subsequent assays challenging bacterial growth.

(2) Does microbial filtrate inhibit Bd *growth?*

Each bacterial isolate was harvested at log phase after 48 hr incubation at room temperature. Plates were rinsed with 2 ml T-broth and the bacterial slurry was filtered through a sterile 0.50 □m syringe-filter to obtain cell-free bacterial filtrate for immediate use in the assay.

(3) Do host peptides inhibit host bacteria?

A subset of bacterial isolates (n=16), each representing a unique OTU, was selected for challenge with host peptides. Since quantities of natural mixtures of skin secretions was limited, only a select subset of bacterial isolates could be tested. Each isolate was cultured for simultaneous *Bd* growth assay (metabolite collection; described above) and bacterial growth assays (growth challenged by host peptides) run in parallel.

Co-culture assays challenging growth of Bd *with bacterial growth*

To screen tadpole-associated skin microbiota for inhibitory activity, we ran all isolates with co-culture assays (Table S2) carried out on media that supported both *Bd* and bacterial growth. Zoospores were harvested and quantified in the methods described earlier and an even lawn transferred to fresh 2% tryptone-RIIA plates and allowed to dry under a sterile hood for 20 minutes prior to streaking the plate with a 5 cm line of each bacterial isolate. Plates were incubated and scored a week later. A distinct zone of inhibition would classify the isolate as inhibitory (Lauer *et al.*, 2007).

Statistical analysis

Both bacterial and *Bd* growth values were determined by first calculating the change in optical density from day 0 to day 7. The mean change in five replicate experiments (wells) was corrected for baseline evaporation by subtracting the mean change in optical density of the negative controls (dead *Bd* or bacteria) from all wells. Growth in a challenge well was calculated as a proportion of growth relative to that observed in the positive control (live *Bd* or bacteria).

RESULTS

Host *Bd* infection status

None of the nineteen adult toads tested positive for the fungal pathogen, *Bd.*

Cultured microbiota

Altogether, 259 bacterial and 113 fungal isolates were cultured from egg clutches and adults. An average of 4.14 bacterial and 3.25 fungal unique morphotype colonies were obtained per individual. All but two individuals yielded culturable growth on both RIIA and SAB media. Another two individuals failed yield fungal growth on SAB (Table S2.2).

Culture-based taxonomic diversity of microbiota

From the cultured isolates, clean sequences were obtained for 213 bacteria and 97 fungi. Sequenced bacterial isolates cultured from *A. obstetricans* adults comprised three phyla, 13 families, 22 genera and 60 unique "species-level" OTUs at the 97% threshold (Table 1). The majority of bacteria isolates belonged to the division *Proteobacteria* (73%). The single largest OTU, no.43, shared by 14 individuals surveyed, was a species of *Pseudomonas fluorescens,* a common biocontrol agent (Nour & El-Ghiet, 2011; Sang & Kim, 2012). The genus *Pseudomonas,* was the most abundant with 51 observations, of which 49% were significantly *Bd*-inhibiting and 9% promoted *Bd* growth.

Of the sequenced fungal isolates, 2 Phyla, 8 orders, 10 families, and 22 OTU species were found at the 97% threshold (Table 2). The most commonly shared OTU, no. 19, a *Mucor sp.* was shared by five individuals and the Mucoraceae family was isolated from 14 of the individuals sampled. It is unknown whether the *Mucor sp.* sequences shared by all life stages and populations in a majority of individuals sampled are commensal opportunists or a potential amphibian pathogen (Shea *et al.*, 1994).

Bacterial OTU diversity

Adult toads in Itingen had more bacterial isolates (n=5) on average than adults from Zunzgen (n=3.5). However, an independent t-test indicated these differences were not significant (t_{16} = -1.56, $p > 0.05$). Similarly, there were more bacteria isolates from adult males carrying eggs (N=4.72 isolates on average) than from adults sampled after norepinephrine administration (N=3.80 isolates on average) to elicit skin defense peptides. However, this was also not a statistically significant difference (t_{18} = 0.633, $p > 0.05$).

Among adults, 38 OTUs were unique to Itingen, 13 OTUs were unique to the Zunzgen population and a small "core" of 8 overlapping OTUs were shared between the two populations. Among egg clutches, 6 were unique to Zunzgen and 4 to Itingen and 1 OTU was shared between populations.

Across life stages, 9 OTUs were unique to the egg clutches, 38 OTUs were unique to adults, and 11 OTUs represent a developmental core between the egg clutches and adults. Specifically for all egg-clutch-carrying adults, at least one isolate was shared between the adult and the egg clutch.

Fungal OTU diversity

While no growth contamination in the water controls was observed among the bacteria RIIA culture, a fungus, sequence matched as *Penicillium expansum* grew in the water control plate. The OTU (no. 15) also matched isolates grown on sample plates. Hence, all thirteen contaminant isolates were expunged from the final analysis. Since fungal spores are airborne and readily spread, it is with some caution that we describe the fungal communities; the extent of symbioses to the host is unknown and merits further investigation. Consequently, on the basis that several OTUs (no.'s 9, 10, 11) also matched *Penicillium expansum* in Genbank and the fact that OTU 9, for example, was the most prevalent OTU (shared by n=15 individuals) surveyed, they too were additionally removed from the final analysis.

Fungal OTU richness ranged from 1-4 species per individual; the mean was significantly lower for adults (n= 1.46) than for egg clutches (n= 2), by independent t-test (t_{19} = -2.64, $p < 0.01$). Across developmental stages, a core of five OTUs were shared, 10 unique to the adult stage and 4 unique to the egg stage. Within adults, a core of 7 different fungal OTUs were shared between populations; 4 OTUs were unique to Itingen and 4 OTUs unique to Zunzgen. Among eggs, 4 different OTUs were shared among populations: 2 were unique to Itingen, 3 unique to Zunzgen. There were no significant differences in richness between OTUs recovered from the two populations for adults (t_{12} = -0.409, $p > 0.05$) or eggs (t_5 = -1.098, $p > 0.05$).

Growth inhibition assays for *Bd* and bacterial isolates

Altogether 53% of bacteria tested significantly inhibited *Bd* growth, 8% promoted growth and 39% neither inhibited or enhanced growth (Table 1, Fig. 2). All adults carried at

least one inhibitory OTU and all but two of the egg clutches carried an anti-*Bd* isolate. The mean proportion of inhibitory OTUs was significantly different between adults (N =0.633) and egg clutches (N=0.575), independent t-test (t_{22} = -2.424, $p < 0.05$). The mean proportion of inhibitory bacterial species between populations did not significantly differ for Intigen (N=0.46) and Zunzgen (N=0.57), (t_{25} = -0.808, $p > 0.05$).

Bd *inhibition for tadpole bacteria*

Of the 123 isolates from tadpoles screened for prospective probiotics, only seven demonstrated inhibition of *Bd* growth via co-culture challenge assays. Pathogen *Bd* inhibition of the filtrate for these isolates was confirmed with quantitative challenge assays formerly described.

Paired growth assays among select OTUs

The sixteen cultured bacteria, representing unique OTU species, selected for paired inhibition assays originated from adult (n=9), egg (n=4), and tadpole (n=1) hosts. Only a single isolate, *Microbacterium* met the traits for an "ideal probiotic": significant inhibition of *Bd* while persisting in the presence of peptides. Of the egg isolates, none inhibited *Bd* but two promoted *Bd* growth. Four bacterial isolates were uninhibited by the host's skin peptides at 400 □g/□L. Unexpectedly, bacterial filtrate for half of the isolates (n=8) promoted *Bd* growth and only two isolates significantly inhibited *Bd* growth.

Skin peptides among life stages

A subset of the described alyteserins present at the metamorph and adult stages were detected in the tadpole stage. No peptides were detected on egg clutches. Sampling of norepinephrine-induced skin peptides from adults from both populations was limited to adults not carrying eggs (n=8) of which three were from Zunzgen and five were from Itingen; rendering a low statistical power to detect differences in peptide profiles between populations.

Table 1. Species-level bacterial OTUs cultured from the skin surface of adult *A. obstetricans* and respective egg clutches at Zunzgen and Itingen.

EMBL Accession Code No.	No. individulas sharing OTU	OTU no.	Phylum	Family	Closest match in GenBank	Population			Life Stage			*Bd* inhibition[a]
						Itingen	Zunzgen	shared	adult	egg clutch	shared	
			Actinobacteria									
HG313721	1	9		Nocardiaceae	Nocardia sp.	X			X			-
HG313726	1	10		Microbacteriaceae	Microbacterium sp.			X	X			-
HF678393	1	25			Rhodococcus sp./ junnanensis / fascians	X			X			-
HG313716	1	45		Micrococcaceae	Arthobacter sp.		X		X			+
HF678395	1	55			Arthrobacter sp.	X			X			
HF678391	1	56			Microbacterium sp.	X			X			-
HG313729	1	58		Streptomycetaceae	Streptomyces sp.	X			X			-
			Bacteroidetes									
HG313690	1	2		Flavobacteriaceae	Flavobacterium sp		X		X			
HG313644					Flavobacterium sp.			X	X			
HG313702	1	6			Flavobacterium sp		X			X		-
HG313663	6	7			Flavobacterium sp	X					X	-
HG313675					Flavobacterium sp	X					X	-
HG313670					Flavobacterium sp	X					X	-
HG313677					Flavobacterium sp.	X					X	-
HE802988					Flavobacterium sp.		X				X	-
HG313731					Flavobacterium sp.						X	-
HG313657	1	14			Chryseobacterium sp.	X			X			-
HG313687	1	15			Flavobacterium sp.	X				X		-
HG313732	1	21			Flavobacterium sp.				X			-
HG313658	2	29			Chryseobacterium sp.	X			X			-
HG313662					Chryseobacterium sp.	X			X			
HG313689	1	30			Flavobacterium sp.		X		X			
HG313660	1	37			Chryseobacterium ureilyticum	X			X			-
HG313637	2	41			Flavobacterium johnsoniae			X	X			-
HG313710					Flavobacterium johnsoniae			X	X			-
HF678400	1	47			Chryseobacterium jejuense	X			X			-
HG313703	1	22			Flavobacterium johnsoniae		X		X			-
HG313673	1	61		Sphingobacteriaceae	Pedobacter sp.	X			X			
HG313681	1	11			Pedobacter sp.	X			X			
HG313715	1	53			Pedobacter sp.			X	X			
HG313678	2	3			Sphingobacterium sp.	X				X		
			Proteobacteria									
HG313682	2	28		Aeromonadaceae	Aeromonas sp.	X			X			-
HF678400					Aeromonas sp.	X			X			-
HG313719	1	31			Pseudomonas sp.	X			X			
HF678390	1	13		Comamonadaceae	Comamonas	X			X			-
HG313728	1	18			Variovorax sp. / paradoxus	X			X			
HG313691	1	33			Comamonas sp.		X		X			
HG313647	2	42			Variovorax paradoxus /sp.	X			X			
HG313652	1	48			Variovorax sp.	X			X			
HG313651	1	4		Enterobacteriaceae	Enterobacter sp.	X			X			
HF678396	1	12			Enterobacter aerogenes	X				X		-
HG313698	2	19			Serratia proteamaculans		X			X		
HG313709					Serratia proteamaculans		X			X		-
HG313697	1	20			Serratia sp.		X			X		+
HG313641	1	23			Rahnella aquatilis	X			X			
HG313683	1	27			Rahnella sp.	X				X		
HF678394	1	35			Citrobacter sp.	X			X			-
HG313646	1	39			Rahnella aquatilis	X			X			
HG313639	1	52			Enterobacter sp.			X		X		
HG313666	2	54			Erwinia persicina	X					X	+
HG313669					Flavobacterium sp			X	X			-
HG313707	3	59			Rahnella sp.			X			X	-
HG313711					Rahnella sp.			X	X			-
HG313725					Rahnella sp.			X	X			
HF678397	1	8		Moraxellaceae	Acinetobacter johnsonii		X		X			-
HG313672	1	26			Acinetobacter sp.	X			X			
HG313706	1	32			Acinetobacter sp.		X		X			
HG313712	1	44			Acinetobacter sp.		X		X			+
HG313674	1	57			Acinetobacter calcoaceticus	X			X			-

[a] *Bd* growth assay outcomes: inhibition (-), growth enhancement (+), or no significant impact on *Bd* growth ( )

Table 1. (*cont.*) Species-level bacterial OTUs cultured from the skin surface of adult *A. obstetricans* and respective egg clutches at Zunzgen and Itingen.

EMBL Accession Code No.	No. individuals sharing OTU	OTU no.	Phylum	Family	Closest match in GenBank	Population			Life Stage			*Bd* inhibition[a]
						Itingen	Zunzgen	shared	adult	egg clutch	shared	
			Proteobacteria (cont.)									
HG313645	1	16		Oxalobacteraceae	Duganella sp.	X			X			-
HG313693	1	1			Janthinobacterium lividum		X			X		-
HG313694	1	51		Pseudomonadaceae	Pseudomonas brassicacearum		X		X			
HG313730	1	5			Pseudomonas sp.	X			X			
HG313654	1	17			Pseudomonas sp.	X				X		-
HG313653	6	24			Pseudomonas filiscindens	X					X	
HG313684					Pseudomonas fluorescens / putida	X					X	+
HG313718					Pseudomonas jessenii			X			X	-
HE802992					Pseudomonas plecoglossicida/alcaligenes	X					X	+
HG313655					Pseudomonas putida	X					X	-
HG313638					Pseudomonas putida	X					X	
HG313642	3	36			Pseudomonas fluorescens	X					X	+
HG313686					Pseudomonas fluorescens	X					X	-
HG313667					Pseudomonas fluorescens strain B79			X			X	-
HG313665	2	38			Pseudomonas putida	X					X	-
HG313659					Pseudomonas sp./ migulae	X					X	
HG313695	7	40			Pseudomonas fluorescens			X			X	-
HG313723					Pseudomonas fluorescens strain B82			X			X	-
HG313707					Pseudomonas gessardii		X				X	-
HG313636					Pseudomonas migulae	X					X	-
HE802991					Pseudomonas migulae	X					X	
HG313685					Pseudomonas sp.	X					X	
HF678398					Pseudoomonas mariginalis						X	
HG313649	14	43			Pseudomonas fluorescens			X			X	+
HG313640					Pseudomonas fluorescens	X					X	-
HG313642					Pseudomonas fluorescens			X			X	-
HG313661					Pseudomonas fluorescens			X			X	-
HG313705					Pseudomonas fluorescens		X				X	-
HG313722					Pseudomonas fluorescens	X					X	-
HG313724					Pseudomonas fluorescens			X			X	-
HG313635					Pseudomonas fluorescens	X					X	
HG313680					Pseudomonas fluorescens			X			X	
HG313668					Pseudomonas fluorescens strain B69			X			X	
HG313696					Pseudomonas fluorescens strain B73			X			X	-
HG313713					Pseudomonas fluorescens strain B77			X			X	-
HG313701					Pseudomonas sp.		X				X	
HG313699					Pseudomonas marginalis		X				X	+
HG313671	8	49			Pseudomonas jessenii			X			X	
HG313717					Pseudomonas koreensis			X			X	-
HG313650					Pseudomonas sp.			X			X	
HG313648					Pseudomonas sp.	X					X	
HG313714					Pseudomonas sp.			X			X	
HG313727					Pseudomonas sp.	X					X	
HG313656					Pseudomonas umsongensis	X					X	-
HG313700					Rhodoccocus sp.			X			X	-
HE802990	2	50			Pseudomonas filiscindens	X					X	
HG313679					Pseudomonas filiscindens	X					X	
HG313664	5	51			Pseudomonas fluorescens	X					X	-
HF678401					Pseudomonas putida	X					X	-
HG313688					Pseudomonas sp.			X			X	-
HG313704					Pseudomonas sp.			X			X	-
HG313692					Pseudomonas sp.		X				X	
HG313676	1	60			Pseudomonas sp./ putida/ fluorescens	X				X		
HG313733	1	46		Shewanellaceae	Shewanella sp.				X			-

[a] *Bd* growth assay outcomes: inhibition (-), growth enhancement (+), or no significant impact on *Bd* growth ( )

Table 2. Species-level fungal OTUs cultured from the skin surface of adult *A. obstetricans* and egg clutches at Zunzgen and Itingen.

No. individuals sharing OTU	OTU ID no.	Phylum	Order	Family	Closest matches in GenBank	Population			Life Stage		
						Itingen	Zunzgen	shared	adult	egg clutch	shared
		Ascomycota									
1	27		Dothideales	Dothideaceae	Aureobasidium pullulans		X			X	
1	2		Hypocreales	Nectriaceae	Fusarium avenaceum		X		X		
4	5		Capnodiales	Mycosphaerellaceae	Cladosporium sp. / cladosporioides			X			X
	5		Capnodiales	Mycosphaerellaceae	Cladosporium sp. / cladosporioides			X			X
	5		Capnodiales	Mycosphaerellaceae	Cladosporium sp. / cladosporioides			X			X
	5		Capnodiales	Mycosphaerellaceae	Cladosporium sp. / cladosporioides			X			X
1	6		Hypocreales	Hypocreaceae	Trichoderma viride/ atroviride	X				X	
1	7		Onygenales	Arthrodermataceae	Trichophyton rubrum	X			X		
1	8		Hypocreales	Hypocreaceae	Trichoderma viride	X				X	
4	13		Hypocreales	Nectriaceae	Fusarium acuminatum			X			X
	13		Hypocreales	Nectriaceae	Fusarium tricinctum			X			X
	13		Hypocreales	Nectriaceae	Fusarium oxysporum			X			X
	13		Hypocreales	Nectriaceae	Fusarium oxysporum			X			X
1	17		Hypocreales	Hypocreaceae	Gibberella avenacea		X		X		
1	18		Helotiales	Sclerotiniaceae	Botrytis sp.			X	X		
1	21		Helotiales	Myxotrichaceae	Geomyces pullulans	X		X			
1	25		Eurotiales	Trichocomaceae	Penicillium sp. FF12	X	X		X		
1	26		Pleosporales	Phaeosphaeriaceae	Ampelomyces sp.				X		
		Zygomycota									
2	1		Mucorales	Mucoraceae	Mucor hiemalis		X			X	
	1		Mucorales	Mucoraceae	Mucor hiemalis		X			X	
4	3		Mucorales	Mucoraceae	Mucor hiemalis			X			X
	3		Mucorales	Mucoraceae	Mucor hiemalis			X			X
	3		Mucorales	Mucoraceae	Mucor hiemalis			X			X
	3		Mucorales	Mucoraceae	Mucor hiemalis			X			X
	4		Mucorales	Mucoraceae	Rhizopus microsporus	X			X		
1	16		Mucorales	Mucoraceae	Mucor flavus		X		X		
5	19		Mucorales	Mucoraceae	Mucor sp.			X			X
	19		Mucorales	Mucoraceae	Mucor sp.			X			X
	19		Mucorales	Mucoraceae	Mucor sp.			X			X
	19		Mucorales	Mucoraceae	Mucor sp.			X			X
	19		Mucorales	Mucoraceae	Mucor sp.			X			X
1	23		Mucorales	Mucoraceae	Mucor sp.		X		X		
1	12				uncultured fungus		X		X		

Table 4. Described skin peptides detected in *A. obstetricans* at different life-history stages. No peptides were detected from swab samples of egg clutches carried by males.

Skin peptide	Sequence	Mono-isotopic mass (m/z)	Signal detected by MALDI MS				Reference
			Egg	Tad.	Meta.	Adult	
Alytesin	pEGRLGTQWAVGHLM-NH2	1535.8	-	X	X	X	Erspamer et al. 1984
Alyteserin-2a	ILGKLLSTAAGLLSNL.NH2	1582.1	-	X	X	X	Conlon et al. 2009
Alyteserin-2c	ILGAILPLVSGLLSSKL.NH2	1605	-	-	X	X	Conlon et al. 2009
Alyteserin-2b	ILGAILPLVSGLLSNKL.NH2	1632.1	-	X	X	X	Conlon et al. 2009
Alyteserin-1c	GLKEIFKAGLGSLVKGIAAHVAS.NH2	2263.5	-	X	X	X	Conlon et al. 2009
Alyteserin-1a	GLKDIFKAGLGSLVKGIAAHVAN.NH2	2277.3	-	X	X	X	Conlon et al. 2009
Alyteserin-1b	GLKEIFKAGLGSLVKGIAAHVAN.NH2	2291.4	-	X	X	X	Conlon et al. 2009
Alyteserin-1d	GLKDIFKAGLGSLVKNIAAHVAN.NH2	2334.5	-	X	X	X	Conlon et al. 2009

Table 5. Abundance of peptides (described and non-described) shared by adult *A. obstetricans* (n=8) surveyed from Zunzgen and Itingen populations (CH). No significant difference between populations was detected for peptide diversity by ANOSIM (r= 3.57, $p < 0.05$) test.

Peptide name	Monoisotopic mass (m/z)	No. adults sharing peptide	Reference
	929	5	
	1042	2	
	1138	4	
	1425	3	
	1498	2	
Alyteserin	1535	6	Erspamer et al. 1984
	1537	2	
	1550	3	
	1569	6	
	1571	5	
Alyteserin 2-a	1583	3	Conlon et al. 2009
	1605	5	
	1614	3	
	1628	4	
	1614	3	
	1628	2	
	1614	3	
	1628	2	
Alyteserin 2-b	1632	6	Conlon et al. 2009
	1632	5	
Alyteserin 1-a	2277	4	Conlon et al. 2009
Alyteserin 1-b	2293	6	Conlon et al. 2009
	2314	4	

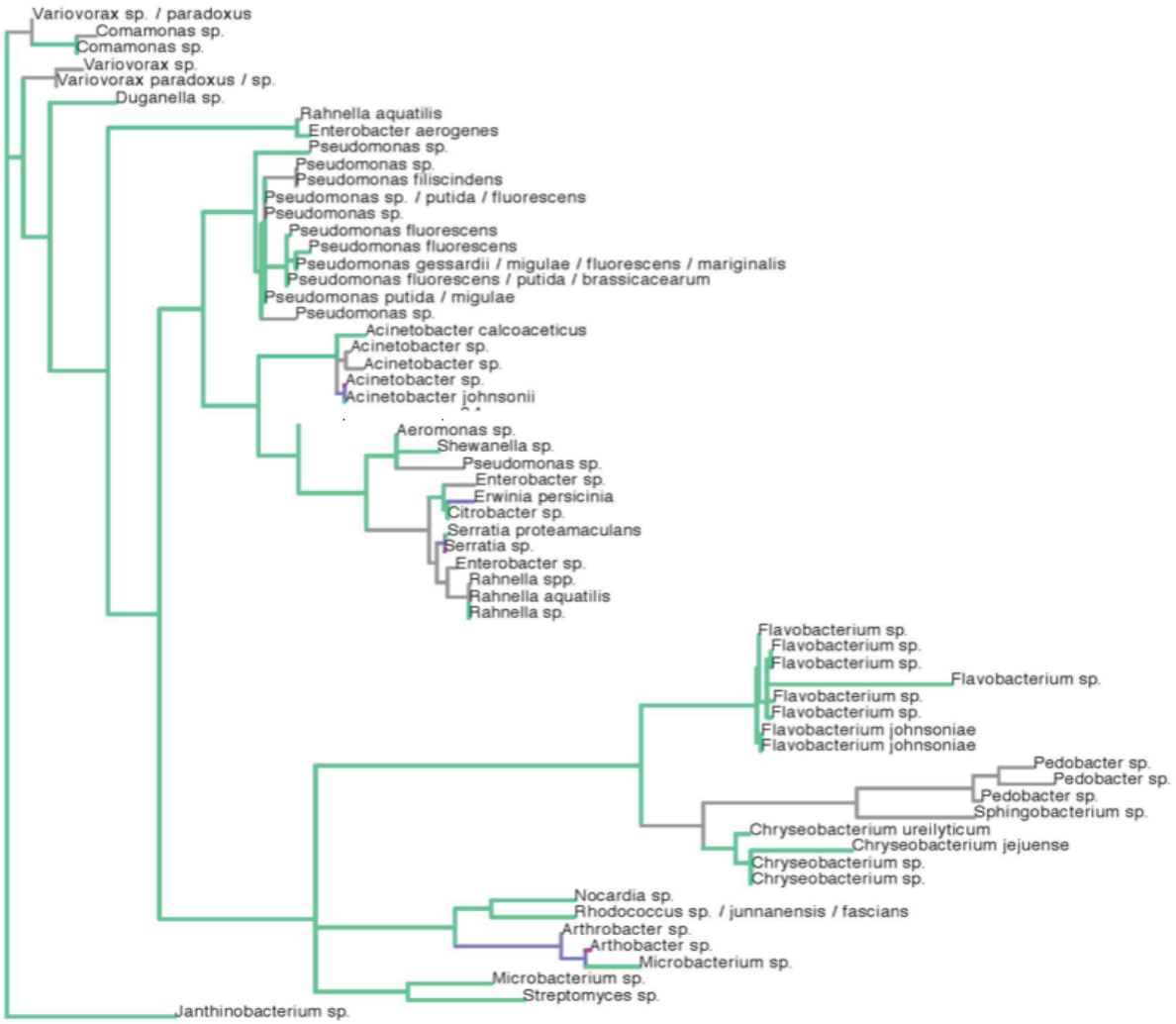

Figure 2. 16s rRNA gene tree of pathogen growth inhibition by each unique OTU in this study. Green lines denote *Bd*-inhibitory isolates, grey lines denote no significant impact on *Bd* growth, and purple lines indicate isolates significantly enhancing *Bd* growth.

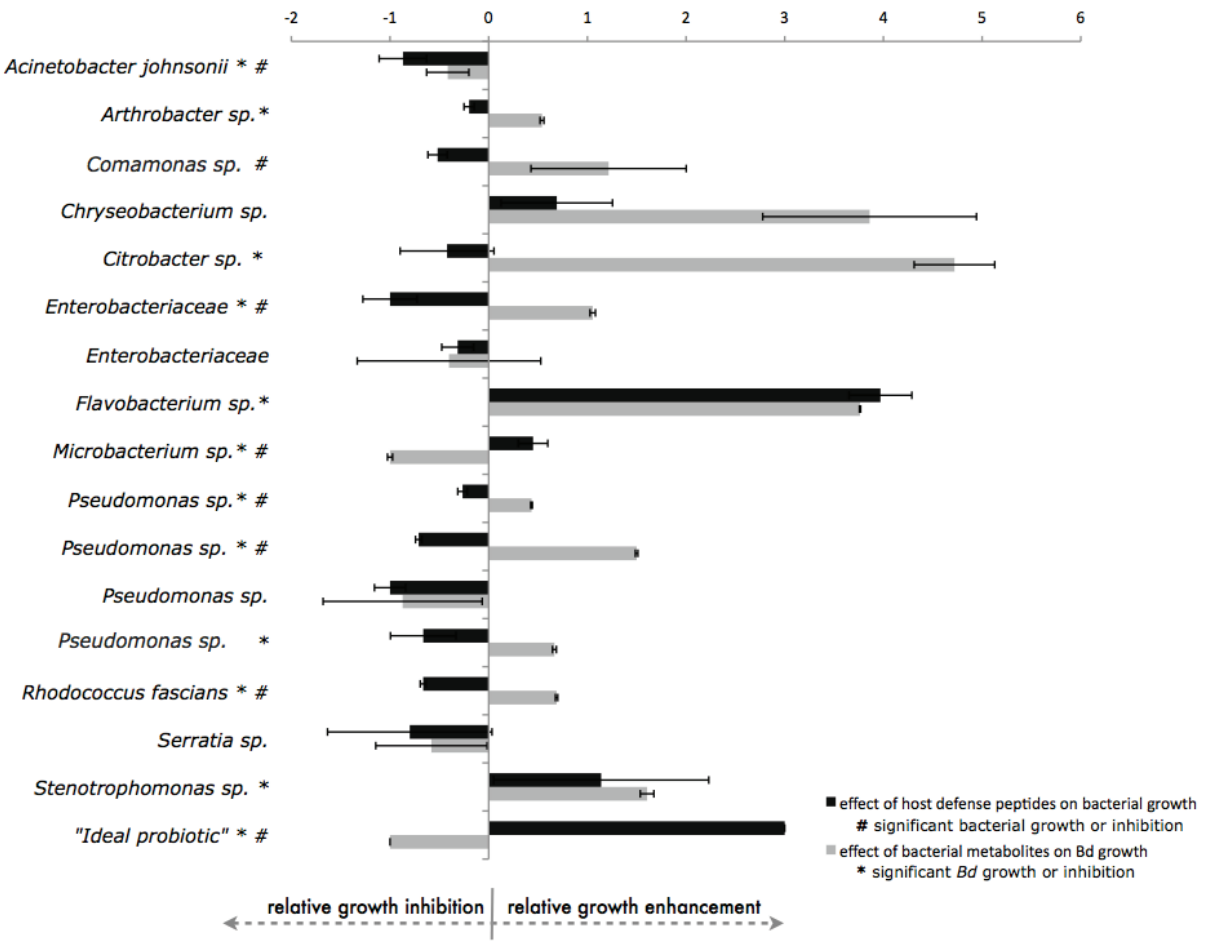

Figure 3. Barplot for paired, relative growth assays of *Bd* growth (grey bars) challenged with bacterial filtrate and bacterial growth (black bars) challenged with *A. obstetricans* AMPs at a fixed concentration of 400 µg/µl. Standard error bars are of a five-well replication in each challenge assay. The * indicates significant Bd inhibition and the # designates significant growth of bacteria determined by a T-Test. Of the bacterial isolates tested (n=16) above, each representing a unique OTU, only the *Microbacterium sp*. satisfied the criteria of an "ideal probiotic": significant inhibition of *Bd* while persisting and having an enhanced growth when challenged by a mixture of granular host peptides.

DISCUSSION

Unraveling complex interactions in any ecosystem is a daunting task. The amphibian skin surface ecosystem is presently in the pioneering phases of discovery, motivated by the amphibian extinction crisis and the role of the skin in immunocompetence. The aim of this study is to provide an immuno-ecological thumbprint for the symbiotic microbial life and defense peptides associated with the skin, and to evaluate microbial community contributions to the stability and disease resistance of the ecosystem. This relies on an accurate profile of the community members (Case, 1990; Frank *et al.*, 2008; Hector & Bagchi, 2007; Logiudice *et al.*, 2003). Here, we first identified the culturable diversity of microbial constituents that contribute to ecosystem equilibrium and help confer disease resistance (Lozupone *et al.*, 2012; Mao-Jones *et al.*, 2010) as a baseline. Next, a probiotic selection protocol specific to species, life history stage, and host population is introduced. We find, through *in vitro* screening, candidates that inhibit pathogen growth through metabolic production.

Despite limitations of the most probable number (MPN) technique for plating culturable microbes (Nannipieri *et al.*, 2003), three phyla were identified in this study. The Bacteroidetes, the Proteobacteria, and the Actinobacteria overlap with dominant phyla reported in other amphibian surveys (Culp *et al.*, 2007; Flechas *et al.*, 2012; Lam *et al.*, 2010; Lauer *et al.*, 2008; McKenzie *et al.*, 2011; Woodhams *et al.*, 2007a). The Firmicutes, present in a variety of amphibian taxa from around the world (Bell *et al.*, 2013; Lam *et al.*, 2010; Lauer *et al.*, 2008; McKenzie *et al.*, 2011; Woodhams *et al.*, 2007b) were not detected among hosts in this survey. However, culture-independent methods reveal Firmicutes is among the most abundant phyla of *A. obstetricans* tadpoles (Davis, 2013). Thus, the absence from the terrestrial phase of the hosts suggests it is either a limitation of culturing methodology or indeed not present. Less diverse were the culturable fungal isolates recovered in this study. We identified fungal species in two sister taxa to the Chytridiomycota: the Zygomycota and the Ascomycota. One impediment to an accurate assessment of amphibian host fungal diversity was due to a lower concentration of DNA than can be extracted in bacteria (Borneman & Hartin, 2000) and as such, not all colonies isolated on SAB media could be extracted for sequencing; casualties in the processing pipeline (Table S1.2). Since the fungi reported herein (Table 2) were cultured from purportedly healthy (*Bd*-free) individuals, the position of these isolates along the continuum from pathogen to saprobe to commensal would need to be assessed.

Most of the fungi represent ubiquitous species found in soil, plants, and the environment (Ghannoum *et al.*, 2010). Yet it is possible for these commensals to predispose to infections, or play an important antimicrobial role in the resident microbial community. One such isolate, an endophytic species of *Ampeloymyces,* commonly forms mutualistic associations with plants by producing metabolites with antimicrobial activity (Aly *et al.*, 2008). Examples of a fungal-vertebrate mutualism are less known. Indeed, amphibian mycology studies often focus on fungal pathogens for the purpose of preventing disease rather than assessing the role of fungi as cutaneous commensals. In addition to *Bd,* some fungal pathogens to amphibians include: *Basdiobolus ranarum* (Taylor *et al.*, 1999), *Amphibiocystidium ranae* (Pereira *et al.*, 2006), *Cladosporium herbarum* (Elkan & Philpot, 1973), as well as a variety of egg pathogens including *Saprolegnia ferax* (Kiesecker & Blaustein, 1995; Kiesecker *et al.*, 2001). None of the fungi identified in this study match described amphibian pathogens, but several isolates within the *Cladosporium* genus were cultivated from eggs and adults. The critical contributions of fungi in soil communities, towards nutrient cycling or as primary producers, are widely documented (Peay *et al.*, 2013). Instances of mutualism with a host are evident: mycorrhiza between fungi and plant roots, (Dickie & FitzJohn, 2007), association with ants, or implementation of fungi as biocontrol. However, to our knowledge, such a mutualism is rarely described between fungal taxa and vertebrate hosts, though many fungi are used as probiotics in plant (Frey-Klett *et al.*, 2011) and in livestock in agriculture (Newbold, 1995).

The absence of *Bd* from all adult toads sampled in this study (N=19) contrasts with a high prevalence at the tadpole stage; in Zunzgen (50%, N=94) and Itingen (45%, N=96), surveyed in parallel to this study (C. Geiger, *unpublished data)*. Yearly surveys since 2007 reveal *Bd* infections of tadpoles at these sites (Tobler, *pers comm)* but none reported in any additional adults (N=37) tested from these populations (C. Geiger, *unpublished data)*. Survival of infected tadpoles observed under laboratory (Tobler & Schmidt, 2010) and semi-natural conditions (Davis, 2013) support population-level differences in host susceptibility to chytridiomycosis through metamorphosis. Our findings corroborate results with microbial fingerprinting techniques (Davis, 2013), indicating population-level differences in taxa richness and diversity between populations and that there are bacterial and fungal species specific to each population. At least one anti-*Bd* isolate was detected in all individuals in this study, whereas only seven of the tadpole bacterial isolates (n=123) tested inhibitory of *Bd*. It is plausible that adults may be better equipped by hosting a higher proportion of anti-*Bd* isolates and this confers a herd immunity effect at the adult stage (Ferrari et al., 2006). The

ability to clear infections at metamorphosis appears linked to survival as an adult. However, since we used different screening methods for isolates at these developmental stages, a study conducted with parallel methods would be more conclusive. All but two of the egg clutches carried an anti-*Bd* isolate. The similarity in OTUs shared between adults and eggs may suggest that adults actively share some of the beneficial bacteria with the egg clutch.

Characterizing a core microbiota of a host (Shade & Handelsman, 2012; Turnbaugh *et al.*, 2007) could have important conservation implications. Predicting the likelihood that a probiotic will colonize the host, or long enough to confer a protective benefit, is an important consideration in candidate selection. One positive indication for community integration is membership in the host's core microbiota; selected probiotics would be shared among populations and across life stages. Isolating potential candidates from healthy individuals of the target host and at the target population is one preliminary step (Klaenhammer & Kullen, 1999) reinforced by limited success of experimental trials with a non-indigenous probiotic, *Janthinobacter lividum* (Becker *et al.*, 2011). Successful trials with the same probiotic (Harris *et al.*, 2009; Vredenburg *et al.*, 2011) were possible in *Rana muscosa* where *J. lividum* was found indigenous to the host microbiota (Woodhams et al. 2007). Curiously, in this survey, the probiotic isolate was isolated from an egg clutch but not shared by any of the adults. While our culture-based survey is limited in depth to characterize a true "core", some insight from the shared microbes can be gathered. One candidate, an anti-*Bd, Pseudomonas fluorescens* (OTU no. 40), for instance, was isolated from both populations and occurs on eggs as well as the adults (Table 1).

The impact of environment, behavior, and diet is underscored in human microbiomes sampled around the world. However, babies from different regions initially harbor similar microbial communities but these communities eventually take on a distinct structure during the early stages of infancy (Yatsunenko *et al.*, 2012). The importance of the first few hours in establishing a microbial community at birth has been documented in mammals (Dominguez-Bello *et al.*, 2010) yet for our host species, the eggs are exposed to a variety of microorganisms prior to being released as tadpoles at the pond site. Determining the stage of initial "inoculum" in *A. obstetricans*— whether it occurs at this point or whether symbionts collected on the egg surface are incorporated in the aquatic life stage— is an important future task towards manipulating mutualistic microbiota through vertical transmission. Compelling evidence for transfer of innate immune defenses have been shown in glass frogs*, Hyalinobatrachium colymbiphyllum*, a species that also exhibits parental care of

embryos (Walke *et al.*, 2011) whereby the male will periodically urinate on the egg clutches (Kubicki 2007) and in the process, transfer defensive peptides. Although no peptides could be detected from the egg clutches of midwife toads in this study, many beneficial antifungal isolates were shared between the male and the egg clutch.

The outcome of bacterial-fungal interactions will vary under different conditions spanning from antagonistic to cooperative (Daskin & Alford, 2012; Frey-Klett *et al.*, 2011). Probiotics may confer a host benefit via various mechanisms to inhibit or reduce the pathogen (Boyle *et al.*, 2006). Likewise, the conditions and principle of the screening method designed to select candidates will simultaneously favor the specific assay's mechanism and conditions, though it is well established that controlled sterile laboratory conditions are vastly different from the fluctuations present on the host mucosal surface. Criteria for a probiotic are also specific to the system (Bletz *et al.*, 2013). In humans, a probiotic to treat Crohn's disease or the digestive microbial flora must meet the important criteria of withstanding a low pH and some antimicrobial conditions (Dunne *et al.*, 2001). Similar to our host, we set our screening temperature to favor pathogen growth at 19°C. While constant temperature is an artificial scenario and it is highly improbable to mimic natural fluctuations in humidity, light, and microbial conditions of amphibian skin; rather for a midwife toad that spends the majority of the time burrowed under a rock crevice, a constant climate may not be too far a departure from nature. The co-culture assays may favor probiotics that operate on competitive exclusion along with metabolic defenses invoked by the pathogen. In contrast, our filtrate method screens strictly on the basis of metabolic products from bacteria for inhibiting pathogen growth. An important consideration for future studies will be determining which combinations of microbes cause beneficial anti-Bd metabolite production that would not otherwise be produced by any microbe in isolation.

Of all the symbiotic bacteria identified and assayed in this study, a majority (53%) produced metabolites that inhibited *Bd* growth (Table 1, Fig. 2). A small proportion of those tested (Fig. 3) are simultaneously capable of withstanding antimicrobial peptides and an even smaller proportion saw unexpected enhanced growth *in vitro* (Fig. 3). Whether competitive exclusion, metabolic warfare, or a coupling of these two mechanisms, are more important mechanisms of effective probiotics in our host, requires further testing. An accurate assessment of the abundance of bacteria on a host's skin (limited by the cultivation methods in this study) is associated with the abundance of metabolites produced to attain immunity (Harris *et al.*, 2006). As we tested bacterial filtrate, our probiotic

recommendations based on this study are limited to those isolates that inhibit *Bd* under controlled conditions. Single isolate impacts on pathogens were assessed and thus we cannot predict the sum outcome of the microbial consortia on *Bd.* Another concern is for how a single-strain probiotic treatment *in vivo* (instead of a consortium) would impact the complex microbial ecology of a host, and how the treatment would deliver a beneficial effect (Dunne *et al.*, 2001).

As diverse as the Amphibia are taxonomically, the peptide cocktail synthesized in the granular glands can be as rich. No two species have identical profiles and an estimated one hundred thousand peptides are expressed by anurans alone (Conlon, 2011b; Vanhoye *et al.*, 2003). Perhaps the most widely recognized and studied function for this suite of peptides is their antimicrobial capacity owing to a common secondary structure, featuring amphipathic (hydrophobic and cationic) amino acid residues. A conserved role as "evolutionarily ancient defense weapons" (Zasloff, 2002) has been widely shown for individual and synergistic peptide defenses against pathogenic bacteria, viruses, and fungi. Extracts of adult *Alytes* peptides inhibited *Bd* at an MIC of 400 □g/□L, a comparatively higher MIC required than among other anurans (Woodhams *et al.*, 2006). Conlon (2011a) proposes an inverse relationship between a species' AMP defense potency and the microbial flora to attain disease resistance. Thus, for species exhibiting a higher AMP potency, a less diverse and abundant microbiota will be present, since natural selection would have acted on those microbes capable to withstand host AMP expression. Vice versa, for species such as *Bufo bufo* that do not express peptides, it is hypothesized that host microbiota would fulfill such antifungal defenses. For instance, in a separate survey with the same culrure protocols employed in this study at a site in northern Switzerland, *Bufo bufo* harbored a higher richness of culturable fungal morphotypes (ranging from nine up to seventeen; Davis, *unpublished observation*) than isolated from the midwife toads in this study. No population-level differences in peptide diversity for adults were detected in this study or for metamorphs from other populations (Davis, 2013). If the peptide repertoire influenced the microbial diversity, it is expected that community fingerprints would be found among populations but this is not our finding. Hence, the initial microbial community established is perhaps more important to community structure over time, though the extent to which peptides regulate diversity is unknown.

Isolating individual peptides expressed by the host permits exploration of potency towards different bacterial (Simmaco *et al.*, 1998). Among the alyteserin peptide families,

subtle differences in primary structure may account for differential antimicrobial potency. For instance, an abundant peptide in this study, alyteserin-2a, is potent against gram-positive bacteria, whereas alyteserin-1a shows better inhibition of gram-negative bacteria (Conlon *et al.*, 2009). Although a majority of the isolates were inhibited by peptides, *Flavobacterium johnsoniae* (OTU no. 22) and *Microbacterium sp.* (OTU no. 56) represent two bacteria isolates with growth enhancement in the presence of the host peptide mixture. This suggests utilization of peptide resources, a hypothesis supported by Daum *et al.,* (2012) for host peptides at low concentrations; a possible scenario when peptides on the skin surface are enzymatically degraded over time (Pask *et al.*, 2012; Resnick *et al.*, 1991). Bacteria mechanisms to resist antimicrobial activity have been proposed (Brogden, 2005; Peschel, 2002): disruption of the membrane, peptide insertion, and tactics to alter the net charge of the peptide (Zasloff, 2002). Gram-negative bacteria (such as the *Flavobacterium*; Fig. 3) reduce susceptibility to AMPs by preventing attachment to the outer membrane. Gram-positive bacteria, including the Actinobacteria cultured in this study, may produce proteolytic enzymes to degrade the antimicrobial peptides. Members of the Actinobacteria isolated in this study were in the genera *Nocardia* and *Rhodococcus*, and may include potential host pathogens. It is possible the *Microbacterium* saw enhanced growth by cleaving the peptides to amino acid residues fueling microbial growth. However, proteolysis is a proposed bacterial mechanism of resistance that requires *in vivo* confirmation (Brogden, 2005). Combined, peptide mixtures provide synergistic effects against a broad-spectrum of bacteria (Hancock & Diamond, 2000). In addition to the host-produced AMPs, metabolites of symbiotic bacteria include lysozyme, proteases, and peptide-based toxins such as bacteriocins thought to inhibit pathogen growth (Bell *et al.*, 2013; Naghmouchi *et al.*, 2012; Rosenthal *et al.*, 2011). Host AMPs may have enhanced pathogen-killing power in synergy with certain bacterial metabolites. Myers *et al.,* (2012) found a synergistic effect between the 2,4-diacetylphloroglucinol metabolite (produced by the cultured isolate, *Pseudomonas fluorescens*) (Brucker *et al.*, 2008) and host peptides that inhibit *Bd* growth at a reduced MIC.

The Pseudomonadaceae comprises nearly half (45%) of all bacterial isolates identified in this study followed by the Flavobacteriaceae, the second most abundant (21%) (Table 1). The Pseudomonadaceae clade also contains the highest number of anti-*Bd* isolates (25 of 51 Pseduomonas isolates tested, a finding supported by previous surveys using co-culture media studies (Flechas *et al.*, 2012; Lam *et al.*, 2010; Walke *et al.*, 2011) as well as quantitative assay methods (Bell *et al.*, 2013). among the bacteria taxa and a higher proportion of anti-*Bd* isolates (16 of the 20 *Flavobacterium* isolates tested inhibitory).

Do biologically active anti-*Bd* compounds relate to taxonomic position? In principle, microbial taxonomic and metabolic diversity are entwined (Jensen & Fenical, 1994), highlighting the importance of screening a range of phylogenetically unrelated candidates for probiotic discovery. However, we find some heterogeneity within taxonomic groups for inhibition and non-inhibition of *Bd* growth (Fig. 2, Table 1), also observed in previous surveys (Flechas *et al.*, 2012). Aside from the Flavobacteriaceae, which consistently yield inhibitory outcomes, the Pseudomonadaceae includes isolates with mixed effects on *Bd* growth (Table S3, Fig. 2, Fig. SX1). Further testing is necessary to explain the minority of non-inhibiting or pathogen-enhancing bacterial filtrate; one component is variation within species and strains. The Pseudomonadaceae comprise 12 OTU-level species, an objective way to classify sequences that matched more than one species (at 99-100%) in the GenBank database. However, strain-level differences have been documented to influence probiotic efficacy (Boyle *et al.*, 2006; Klaenhammer & Kullen, 1999).

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SUPPLEMENT

Accounting for block-effects among micro-well plates in challenge assays

A pilot trial was initially conducted to determine if there are block-effects in assay outcomes owing to microhabitat differences between blocks due to light or temperature gradients. In the trial, five 96-microwell plates were prepared with five different bacterial filtrate-*Bd* growth challenges. The plates were stacked and allowed to grow for a week at 19 degrees Celsius. No differences in inhibition and enhancement activity was found among isolates tested across the five blocks, regardless of placement in the stack.

We concluded that assays run in parallel could have comparable results; possibly assisted by maintaining a perimeter of T-broth only and concentrating the "challenge" wells at the center of the plate and consequently minimizing potential evaporation effects or contaminants that may supersede the parafilm seal.

How to account for differences in activity among bacteria belonging to an OTU and isolated from the same individual (Fig. SX1, Table S3)

30 OTU isolates (listed in Table S3) were genetic replicates for isolates cultured from the same individual. Although they were selected as morphologically distinct colonies (or in the case of ambiguity, veered on the side of a distinct isolate), they share 97% similar genetic material and thus form genetic replicates within an OTU. Among these replicates, for 7 OTUs, duplicate assays yielded consistent outcomes: either inhibition, enhancing, or non-significantly inhibiting or enhancing outcomes (listed in the table as "I", "E", or "NS". The other 23 OTU replicates have a combination of assay outcomes: I, NS, or E. Determining these discrepancies in outcomes require further testing. It is possible there are strain-level differences within an OTU that may account for different inhibition effects or context-dependency among isolates cultured on different days and from different plates.

Table S1.1 Sampling summary for *A. obstetricans* defenses, *Bd*, and microbiota from adults and egg clutches

Individual no.	(A)dult, carrying (E)ggs, egg (C)lutch	population	sex (M)ale	weight (g)	norepinephrine (NE) peptide collection or blot	bacteria culture	fungal culture	*Bd* swab	location of collection
1	A	Itingen	na	7.77	NE	RIIA	SAB	x	hilside above pond
2	A	Itingen	na	6.09	NE	RIIA	SAB	x	hilside above pond
3	A	Itingen	na	7.99	NE	RIIA	SAB	x	hilside above pond
4	A	Itingen	na	7.05	NE	RIIA	SAB	x	hilside above pond
5	A	Itingen	na	8.8	NE	RIIA	SAB	x	hilside above pond
6	A	Zunzgen	na	8.48	NE	RIIA	SAB	x	tree stump
7	A	Zunzgen	na	7.45	NE	RIIA	SAB	x	tree stump
8	A	Zunzgen	na	8.19	NE	RIIA	SAB	x	tree stump
9	E	Itingen	M	na	blot	RIIA	SAB	x	upland in leaves and rocks
10	E	Itingen	na	na	blot	RIIA	SAB	x	upland in leaves and rocks
11	E	Itingen	M	na	blot	RIIA	SAB	x	upland in leaves and rocks
12	E	Itingen	M	na	blot	RIIA	SAB	x	road xing
13	E	Itingen	M	na	blot	RIIA	SAB	x	spring/creek side
14	E	Itingen	M	na	blot	RIIA	SAB	x	spring/creek side
15	E	Itingen	M	7.51	blot	RIIA	SAB	x	spring/creek side
16	E	Zunzgen	M	na	blot	RIIA	SAB	x	tree stump
17	E	Zunzgen	M	na	blot	RIIA	SAB	x	tree stump
18	E	Zunzgen	M	na	blot	RIIA	SAB	x	tree stump
19	E	Zunzgen	M	na	blot	RIIA	SAB	x	tree stump
20	C	Itingen	na	na	blot	RIIA	SAB	na	upland in leaves and rocks
21	C	Itingen	na	na	blot	RIIA	SAB	na	upland in leaves and rocks
22	C	Itingen	na	na	blot	RIIA	SAB	na	road xing
23	C	Itingen	na	na	blot	RIIA	SAB	na	creek side
24	C	Itingen	na	na	blot	RIIA	SAB	na	creek side
25	C	Itingen	na	7.51	blot	RIIA	SAB	na	creek side
26	C	Zunzgen	na	na	blot	RIIA	SAB	na	tree stump
27	C	Zunzgen	na	na	blot	RIIA	SAB	na	tree stump
28	C	Zunzgen	na	na	blot	RIIA	SAB	na	tree stump
29	C	Zunzgen	na	na	blot	RIIA	SAB	na	tree stump

Table S1.2 Sampling summary for *A. obstetricans* surface defenses, pathogen, and microbiota from adults and egg clutches

		Fungi			Bacteria				
Individual no.	(A)dult, carrying (E)ggs, egg (C)lutch	original morphotypes	reiteration	no. 18S OTUs	original morphotypes	reiteration	no. 16S OTUs	no. inhibitory 16S OTUs	proportion of OTUs on individual that are inhibitory
1	A	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth
2	A	no growth	no growth	no growth	1	1	1	1	1.00
3	A	3	8	3	5	8	4	3	0.75
4	A	3	2	2	3	5	4	3	0.75
5	A	no growth	no growth	no growth	3	5	4	1	0.25
6	A	5	8	1	6	16	9	5	0.56
7	A	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth
8	A	5	3	1	3	5	1	1	1.00
9	E	2	2	2	5	14	4	3	0.75
10	E	2	2	1	6	17	7	3	0.43
11	E	2	2	2	3	6	3	1	0.33
12	E	4	3	0	3	9	6	1	0.17
13	E	2	3	2	5	12	7	5	0.71
14	E	4	4	2	5	34	8	3	0.38
15	E	2	5	2	4	13	7	3	0.43
16	E	1	1	1	5	6	4	1	0.25
17	E	3	11	3	2	11	4	2	0.50
18	E	4	2	2	6	7	1	1	1.00
19	E	5	8	3	3	10	4	3	0.75
20	C	6	2	3	7	9	3	1	0.33
21	C	5	8	2	5	7	1	0	0.00
22	C	1	1	0	3	5	3	3	1.00
23	C	5	6	3	6	13	4	3	0.75
24	C	no growth	no growth	no growth	3	5	4	1	0.25
25	C	4	19	6	4	12	6	3	0.50
26	C	2	2	0	2	2	2	0	0.50
27	C	3	6	3	3	8	3	1	0.33
28	C	1	2	2	6	6	2	1	0.50
29	C	4	8	2	5	15	3	2	0.67

Table S2. Summary of growth inhibition assays for *Bd* and bacterial isolates challenged with skin peptides and bacterial filtrate.

			Growth factor			Challenge factor				
Assay Type	Assay No.	challenge type	growth inhibition factor	quanitfication of factor	concentration of factor introduced	challenger factor	quantification of challenger	concentration of challenger introduced	no. assays run	purpose
microwells; spectrophotometric absorbance										
	1	1 vs.1	*Bd*	Yes	no. zoospores	bacteria filtrate	No	filtrate for bacterial isolate	276	for all unique morphotypes from *Bd*-free adults and egg clutches
	2	1 vs. mixture	*Bd*	Yes	no. zoospores	peptides	Yes	peptide concentration	5	to determine MIC standard for AMP to inhibit *Bd*
	3	1 vs. mixture	bacterial growth	No	stdd preparation of bacteria cultures	peptides	Yes	peptide concentration	16	paired assays; limited by peptides extracted
co-culture; zone of inhibition										
	4	1 vs. 1	*Bd*	No	stdd preparation of bacteria cultures	bacteria	No	bacteria streak	123	high-throughput screening of anti-*Bd* bacteria among infected tadpoles

Table S3. Bacteria filtrate yielding consistent or mixed effects on *Bd* growth for assay replicates of an OTU found on an individual.

OTU no.	NCBI sequence matches	no. of individuals with this OTU	no. replicates for this OTU on an individual (x,y)	Effect of OTU on *Bd* growth: (**E**)nhancing, (**I**)nhibiting, (**NS**)Non-significant, or a mixture (**IENS**).	total no. replicates for an OTU
OTUs yielding a consistent effect on Bd *growth among replicates*					
8	Acinetobacter johnsonii	1	2	**I**	2
44	Acinetobacter sp.	1	2	**E**	2
28	Aeromonas sp.	1	2	**I**	2
2	Flavobacterium succinicans	1	2	**NS**	2
7	Flavobacterium sp.	2	2,2	**I**	4
40	Pseudomonas gessardii	1	2	**I**	2
17	Pseudomonas sp.	1	2	**I**	2
OTUs yielding mixed effects on Bd *growth among replicates*					
36	Pseudomonas fluorescens strain	1	6	**IENS**	12
38	Pseudomonas fluorescens strain B79	1	6	**INS**	6
39	Pseudomonas putida	1	3	**INS**	3
41	Rahnella aquatilis	1	2	**IE**	2
43	Pseudomonas fluorescens strains / B73 / B69	8	2,2,2,2,2,4,5,6,4	**IENS**	29
47	Chryseobacterium jejuense	1	2	**INS**	2
49	Rhodoccocus sp / Pseudomonas umsongensis	3	2,2,2	**INS**	6
51	Pseudomonas chloraphis / putida / sp.	3	2,3,2	**INS**	7
54	Erwinia sp.	1	4	**IENS**	4
59	Rahnella sp.	1	3	**IENS**	3

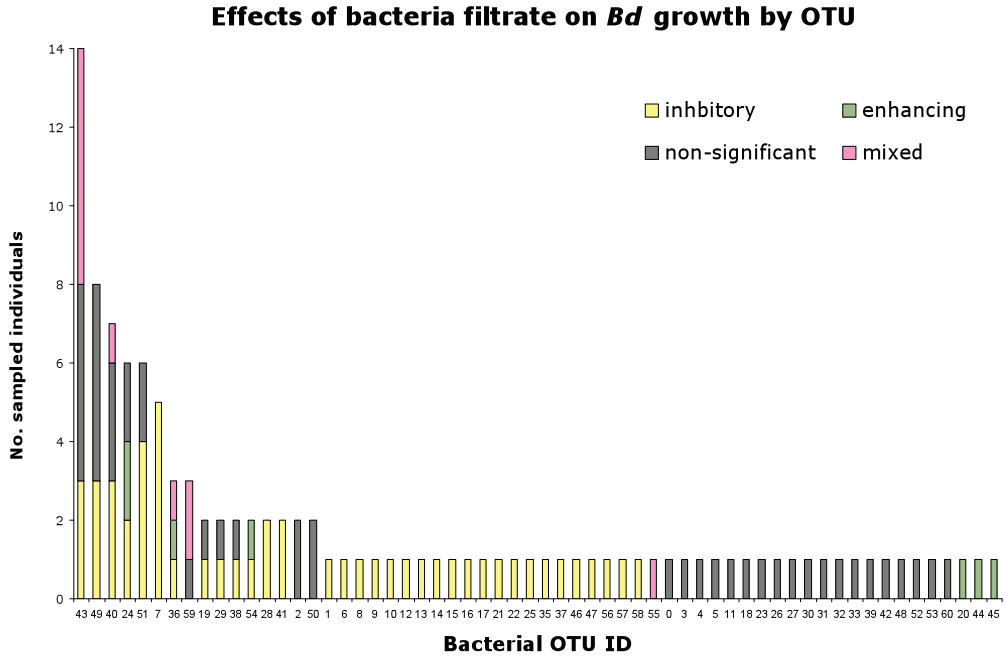

Figure SX1. Mixed outcomes on *Bd* inhibition for a given OTU sampled among individuals.

CHAPTER TWO

Trajectories of amphibian microbiota: response to probiotic therapy depends on initial community structure

Leyla R. Davis, Laurent Bigler, and Douglas C. Woodhams

ABSTRACT

The holobiome, or combined genomes of the host and symbionts, can shift with changing environments, affecting host health. Probiotic therapies aim to improve host health by altering the microbiota. There is currently little understanding of how initial microbial community structure affects trajectories of the holobiome. We conducted bacterial therapy trials with 240 aquatic larvae of the midwife toad, *Alytes obstetricans,* collected from four populations in Switzerland. All tadpoles were naturally colonized by the fungal pathogen *Batrachochytrium dendrobatidis*, agent of the emerging disease chytridiomycosis, and a leading cause of global amphibian population declines and extinctions. Tadpoles from each population were treated with probiotic baths of *Pseudomonas fluorescens, Flavobacterium johnsoniae*, or control inoculations under semi-natural conditions in outdoor mesocosms, and sampled through metamorphosis. Bacterial assemblages were compared at the time of tadpole collection from ponds, post-treatment, at metamorphosis, and at the end of the summer growing season by the culture-independent microbial fingerprinting technique, terminal-restriction fragment length polymorphism (T-RFLP). Fungal assemblages were examined at the tadpole stage. A subsample of tadpoles exposed to *F. johnsoniae* and controls were analyzed additionally by shotgun metagenomics to identify microbiota function and membership in greater depth. Microbial community structure differed significantly among host populations and treatments at the tadpole stage and through metamorphosis. We describe a core microbiota defined by persistence through development and by shared membership across populations. A correlation between host skin peptides and microbiota suggests a potential mechanism of host-directed symbiosis throughout development. Early developmental stages are ideal targets for probiotic therapy that can govern the trajectories of microbiota.

***Keywords*:** *Alytes obstetricans,* population, development, MADLI-MS, holobiome, T-RFLP, shotgun metagenomics

INTRODUCTION

Symbiotic relationships between host and microbes are common (Koch & Schmid-Hempel, 2011; Leff & Fierer, 2013; Rosenberg & Zilber-Rosenberg, 2011; Rosenthal *et al.*, 2011a). There is currently little understanding of the factors underlying changes in the composition or function of microbiota (Cho *et al.,* 2012; Lozupone *et al.,* 2012; Yatsunenko *et al.,* 2012), or the relative roles of environment or host immunity in regulating microbiota (Bevins & Salzman, 2011; Charlotte Jander *et al.*, 2012; Leff & Fierer, 2013). The composition of the mouse gut microbiota for instance, is affected by early life events (Cho *et al.,* 2012), host genotype (Ley *et al.,* 2008), and maternal inheritance (Dominguez-Bello *et al.*, 2010; Ley *et al.*, 2005). Vertical transmission, i.e. transmission from parent to offspring, of protective microbiota has been suggested to occur in amphibians as well (Walke *et al.,* 2011). Amphibian hosts of the same species living in different aquatic habitats have more similar microbial community compositions than cohabiting hosts of different amphibian species (McKenzie *et al.,* 2011), indicating strong host regulation of symbiotic microbiota.

Our purpose in this study is to describe the variability in the microbiota of a single host species among populations and through life-history as well as to test for effects of probiotic applications that purposefully introduce changes in the microbiota. We measure how factors including host population of origin or treatment with probiotics influence the trajectory of the microbial community through culture-independent techniques. Information from bacterial 16S rRNA and fungal ITS genes provides information on the structure and membership of microbial communities. We use terminal-restriction fragment length polymorphism (T-RFLP) to obtain a fingerprint of microbial community structure and membership through each resulting digest fragment or phylotype. Shotgun metagenomics provides deeper insight into microbial species composition, relative abundance, and microbiome function.

Symbiotic host associations are important in infection resistance and tolerance, among other functions (Wilson, 2005). Inhibition of pathogens by symbionts may be specific as shown by invertebrate pathogen defenses (Dillon & Dillon, 2004; Koch *et al.*, 2012) or a general function of the microbial community (van Elsas *et al.*, 2012; Wilson, 2005). For example, microbial profiling of corals provides a powerful tool for identifying reefs vulnerable to disease outbreak (Klaus *et al.,* 2007). The gut of *Anopheles* mosquito larvae hosts microbiota that can directly kill *Plasmodium* and other parasites or interact with host defenses (Gonzalez-ceron

et al., 2003). Bumblebees have a unique microbiota often explained by colony affiliation (Koch & Schmid-Hempel, 2011) and the 'type' of microbial resident community influences the specificity of host-parasite interactions (Koch *et al.,* 2012). The role of amphibian skin microbiota in defense against an emerging fungal disease, chytridiomycosis, is becoming a research focus for conservation biologists and ecologists (Bletz *et al.,* 2013).

Our focal host is the common midwife toad, *Alytes obstetricans*. This species has become a model for chytrid studies among amphibians across Europe (Bosch *et al.,* 2001), with remarkable among-population variation in susceptibility to the fungal agent *Batrachochytrium dendrobatidis* (*Bd*) causing the disease (Tobler & Schmidt, 2010a). Here, our focus is on understanding the host microbiome, a trait considered to be an extension of the host's immune system (Woodhams *et al.*, 2007b). Adaptive and innate immune responses of amphibians have been well described (Rollins Smith & Woodhams 2012). Among these sophisticated defenses, host antimicrobial peptides (AMPs) have been suggested to help regulate the microbiota of amphibians (Conlon, 2011a). While the present paradigm for host peptide-microbial interactions centers on the manufacture of broad-spectrum antimicrobial peptides mounted as a defensive response, recent studies indicate that the mucosal layer of many amphibians contains ambient levels of defense peptides (Pask *et al.,* 2012; Woodhams *et al.,* 2012). We found that the growth of some symbiotic bacteria isolated from *A. obstetricans* is inhibited while others are enhanced by skin defense peptides (L.R.D. & D.C.W., unpublished). Here we test for potential innate immune defense interactions between ambient host AMPs and microbiota

By monitoring the trajectory of microbial diversity among different populations and through development, we describe stable, or core components (Roeselers *et al.*, 2011; Turnbaugh *et al.*, 2007) of the microbiome associated with amphibian skin. We also test how manipulations of the microbiota through probiotic treatments at the tadpole stage can affect community structure, function, and abundance. We examine skin defense peptides as a contributing factor explaining variation, and thus a potential regulatory mechanism for skin microbiota.

MATERIALS & METHODS

Animals

Sixty *A. obstetricans* tadpoles were collected from each of four different ponds in Switzerland in early May 2011. For pond descriptions see Supplementary Information (Table S1). Populations were selected based on the following criteria: (1) confirmed *Bd*-positive in previous surveys (Garner *et al.*, 2005; Tobler *et al.*, 2012b; Tobler & Schmidt, 2010a) (2) are geographically disparate locations and (3) the ponds sustained relatively large populations of overwintered tadpoles that had not yet reached Gosner (1960) Stage 42 (i.e. with denticles still intact). Permits to conduct fieldwork were obtained from the cantonal conservation authorities of Basel-Landschaft, Luzern, St. Gallen, and Zürich; all animal procedures were approved by the Veterinary Authority of Zurich (227/2007) and the Federal Office for the Environment. After collection, tadpoles were temporarily stored in 20 L tubs in the lab in order to confirm infection status by rt-PCR according to Boyle *et al.,* (2004) before being placed in outdoor semi-natural conditions for the experiment. Uninfected metamorphs were returned to their pond of origin upon completion of the experiment according to permit guidelines.

Bacterial isolation and culture

Two bacterial strains, *Pseudomonas fluorescens* and *Flavobacterium johnsoniae,* were originally isolated from the rinsed skin surfaces of tadpole and adult *A. obstetricans* captured from Zunzgen in Kanton Basel in May 2009 and selected out of approximately 250 isolates collected (L.R.D. & D.C.W., unpublished). Isolates were screened for ability to inhibit *Bd* growth and capacity to resist *A. obstetricans* AMP secretions *in vitro*. We selected *P. fluorescens* based on: (a) high prevalence; isolated from 12 of 19 adult hosts examined in the survey, (b) potential to persist across generations and occur in amphibian hosts from Australia and from North and Central America (Lauer *et al.*, 2008b; Walke *et al.*, 2011; Woodhams *et al.*, 2007b) and (c) common implementation as a biocontrol in agriculture, due to a highly conserved, 2,4 DAPG antifungal metabolite, (Keel *et al.,* 1996). As probiotic inoculation would take place at the tadpole stage in this trial, a second probiotic, *F. johnsoniae,* was selected because it was commonly isolated from *A. obstetricans* tadpoles. This common freshwater Gram-negative bacterium is also present in soil where it rapidly degrades chitin (McBride *et al.,* 2009) and certain strains have shown antifungal activity, a promising character for a biocontrol agent against *Bd* (Sang & Kim, 2012). Cultures were prepared on low nutrient RIIA agar (BD Difco™) plates under a laminar flow hood and incubated for 72 hr at room temperature. Ten

plates per probiotic were each rinsed with 2 ml MiliQ water, incubated 5 min, and rinsed with an additional 5 ml MiliQ water and combined before treatment.

Experimental design

The experiment was conducted under semi-natural conditions in outdoor mesocosms (Wilbur, 1997) using standard methods (Buskirk, 2002). The experiment had two factors: probiotic therapy and population of tadpole origin. There were three levels of probiotic treatment (control, treatment with *F. johnsoniae* and treatment with *P. fluorescens*) and four levels of population of origin (Zunzgen, Altstätten, Schauensee, and Gamlikon), yielding 12 treatment combinations. A total of 48 mesocosm tubs (each 0.28 m^2, 80 L) were set-up on April 27, 2011 to contain 10 g of dried beech *(Fagus sylvatica*) leaf litter, 2 g of rabbit food, and 1 cup of diverse zooplankton in 60 L of aged tap water. A screen mesh protected tadpoles from direct sunlight and predator disturbance. On May 16, groups of five tadpoles from each of the four populations were randomly distributed into each of the 48 tubs (Fig. S1). Tadpoles were acclimated to outdoor tub conditions for one week and then inoculated with either the probiotic bacterium or sterile medium by bathing for 2 hr in 2 mL probiotic slurry before being poured back into their mesocosms with the bacterial slurry. Groups of five tadpoles were inoculated with either *P. fluorescens* (n=12) or the sterile media control (n=12) on May 21, or with either *F. johnsoniae* (n=12) or sterile media control (n=12) on May 23. Control treatments consisted of rinses of sterile RIIA agar plates.

Upon metamorphosis, tadpoles were collected with a tank-specific net and housed in an autoclaved 1 L plastic tub containing 200 mL of the mesocosm's tub water and several beech leaves for shelter. These tubs were arranged on shelving in keeping with the block design and rotated 2x weekly within the respective block. All tubs were tilted to simultaneously provide land and water access for the late-stage tadpoles and terrestrial toadlets. Frogs were fed with crickets dusted with calcium three times weekly and the water changed the day after feeding. The experiment was completed one week following the last individual's metamorphosis, on August 8.

Microbiota sampling

Microbiota were sampled with a sterile plastic rayon swab (COPAN Italia, S.p.A., Brescia, Italy) at three time points. At the first two time points, we swabbed the buccal cavities of tadpoles with denticles (i.e. pre-Stage 42) upon collection from ponds and one week following inoculations (May 28 & 30). At the last time point, we swabbed skin surfaces directly upon completion of metamorphosis (Stage 46). The entire body surface was swabbed beginning with each of the hindlimb and forelimb digits, flanks, and the ventral/dorsal surfaces (10x each). Separate pairs of gloves and paper cups were used when handling each tadpole or metamorph to avoid contamination. Bias in swabbing technique was reduced by having a single swabber to ensure consistent capture of microbiota. Microbial swabs were stored at -20°C prior to DNA extraction with a QIAGEN DNeasy Blood & Tissue Kit (Hombrechtikon, Switzerland) according to the manufacturer's protocol including a lysozyme incubation step. Buccal swabs of tadpoles with denticles (before stage 43) were pooled by mesocosm and extracted, and swabs of metamorphs at stage 46 were extracted individually. From the 60 □l eluted DNA extract, *Bd* infection loads and microbiota diversity were analyzed with two methods: real time polymerase chain reaction (rt-PCR) and T-RFLP respectively. A subsample was also analyzed by shotgun metagenomics. Detailed methodology can be found in the Supplementary Information.

Microbiota analyses

To obtain a deep sequencing of the tadpole buccal microbiome, a subsample of six mesocosms (30 tadpole swabs) of which half of the tubs were treated with *F. johnsoniae* and the other tubs of control tadpoles, were analyzed by shotgun metagenomics. Initial processing including quality control was performed under metagenome rapid annotation using subsystem technology (MG-RAST v3.2.5.2; /http://metagenomics.anl.gov/) where the data have been made publically available. Best-hit classification was run with the M5 non-redundant protein database with a 60% min identity cutoff, 45 bp min alignment length cutoff, and E value of 10^{-10} and constrained to 8223 bacterial sequences (Table S2). Visualization of bacterial composition at the genus level was obtained with Krona, and a representative diagram is presented (Fig. 1). See Figure S3 for diagrams of all six metagenomes at the phylum level. Statistical comparisons of taxa abundance between treatments were performed with MG-RAST. Environmental gene tags were examined to compare bacterial community function between treatments using the subsystems protein database for hierarchical classification. Functional abundance was visualized by

principle coordinates analysis, and further analyzed by comparing unique and shared functions between treatments based on KEGG mapping.

Culture independent microbiota were obtained with T-RFLP and the data analyzed in PAST (Hammer *et al.,* 2001), using ANOSIM (Analysis of Similarity) and NPMANOVA (Non-Parametric Multivariate Analysis of Variance) with the Bray-Curtis similarity matrix. We examined the factors population, treatment, and their interaction for effects on microbial community. Data were visualized with nMDS (non-Metric Multidimensional Scaling). We present T-RFLP results from analyses using Msp1 and Hae3 enzyme digests. Comparisons of enzyme efficiency are provided in the Supplementary Information.

Skin peptide profiling

To profile skin peptide composition, all surviving metamorphs (n=60) were swabbed on the last day of the experiment using a cotton swab (Schaffhauser-Neuhausen, CH). Each swab was swirled for 2 min in a 1.5 mL tube (Eppendorf) containing 1 mL of 90% methanol. Peptides were dried in a rotary evaporator at 60°C, weighed, and stored at -20°C. Peptide samples were analyzed with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS, Bruker Autoflex I, Bruker Daltonics GmbH, Bremen, Germany). To minimize potential variation among runs, all samples were run on the same day, using the same target, and same reagents. To avoid potential contaminants, only Eppendorf and Lo-Retention (GmBH) pipette tips were used. Dry peptide samples were reconstituted in 30 µl of a 2:1 concentration of HPLC-grade water and acetonitrile with 0.2% trifluoroacetic acid and vortexed 5 sec prior to applying 0.5 µl of peptide solution to a pre-spotted AnchorChip (Bruker) target prepared with α-cyano-4-hydroxycinnamic acid as matrix (HCCA, Bruker). After 2 min of evaporation, peptides were rinsed with 7 µl of 10 mM aqueous ammonium dihydrogen phosphate buffer containing 0.1% trifluoroacetic acid. A mixture of peptide standards was used to calibrate the instrument within the target mass range, 800-3000 *m/z*. The peptide calibration II standards (Bruker) included: bradykinin (*m/z* 757.40), angiotensin II (*m/z* 1046.54), angiotensin I (*m/z* 1296.69), rennin substrate (*m/z* 1758.93), and ACTH clip 18-39 (*m/z* 2465.20). Spectra averaging 300 total shots representing 30 shots each taken from various locations on the target were exported to Flex Analysis (v3.3 Bruker Daltonics, GmBH) and processed. Peaks were smoothed and mass lists determined with the SNAP method of peak detection, a signal to noise ratio of 2, and TopHat baseline subtraction method. Spectra were exported to Excel v.12.1.0 and standardized by computing peak intensity relative to an intrinsic peak standard,

1605 *m/z*, present in every sample. Peptide profiles were analyzed by ANOSIM for differences among populations or treatments. To test for associations between individual peptides and individual microbial phylotypes at the skin surface, a pairwise regression correlation test was run in R Studio and all *p* values FDR-corrected (Benjamini & Hochberg, 1995) as implemented by Garcia (2004). Mean peptide profile and T-RFLP profile per mesocosm were used as independent samples. Finally, a canonical correspondence analysis (Hammer *et al.,* 2001) was used to assess microbial phylotype variation as a function of the skin peptide expression.

RESULTS

Microbial composition and community structure

Of the 8223 bacterial sequences analyzed by shotgun metagenomics, approximately 85% belonged to the Proteobacteria (Figs. 1, S3). The distribution of genera for a mesocosm tub representative (containing 5 control tadpoles) is shown in Figure 1. Several commonly cultured bacteria used for probiotic experiments in adult amphibians in this and other studies (e.g., Harris *et al.,* 2009a,b, Woodhams *et al.,* 2012) occurred on the *A. obstetricans* tadpoles including *Pseudomonas*, *Flavobacterium*, and *Janthinobacterium*. However, these were minor components of the community.

T-RFLP analysis of bacterial communities on tadpoles from four populations demonstrated distinct community composition in field-collected samples (Fig. 2a). These population-level signatures persisted after placement of tadpoles into standardized mesocosm conditions and through treatment with probiotics (Fig. 2b). After metamorphosis, a significant population-of-origin signal remained detectable (Fig. 2c). Results of NPMANOVA (Table 1) and ANOSIM (Table S4) indicate strong population effects on bacterial communities. In field-collected tadpoles, fungal communities were also distinct among the four populations (Fig. 2d, Table 2). We used the *R* statistic from ANOSIM to describe the similarity of the microbiota (Clarke, 1993; Rees *et al.*, 2004). Among wild pre-treated populations, the *R* statistic is 0.8033 followed by post-treatment at 0.4342, and upon metamorphosis, 0.1244, indicating a population's microbiota were increasingly more similar to one another through development and through time in captivity (Fig. 2, Table S4).

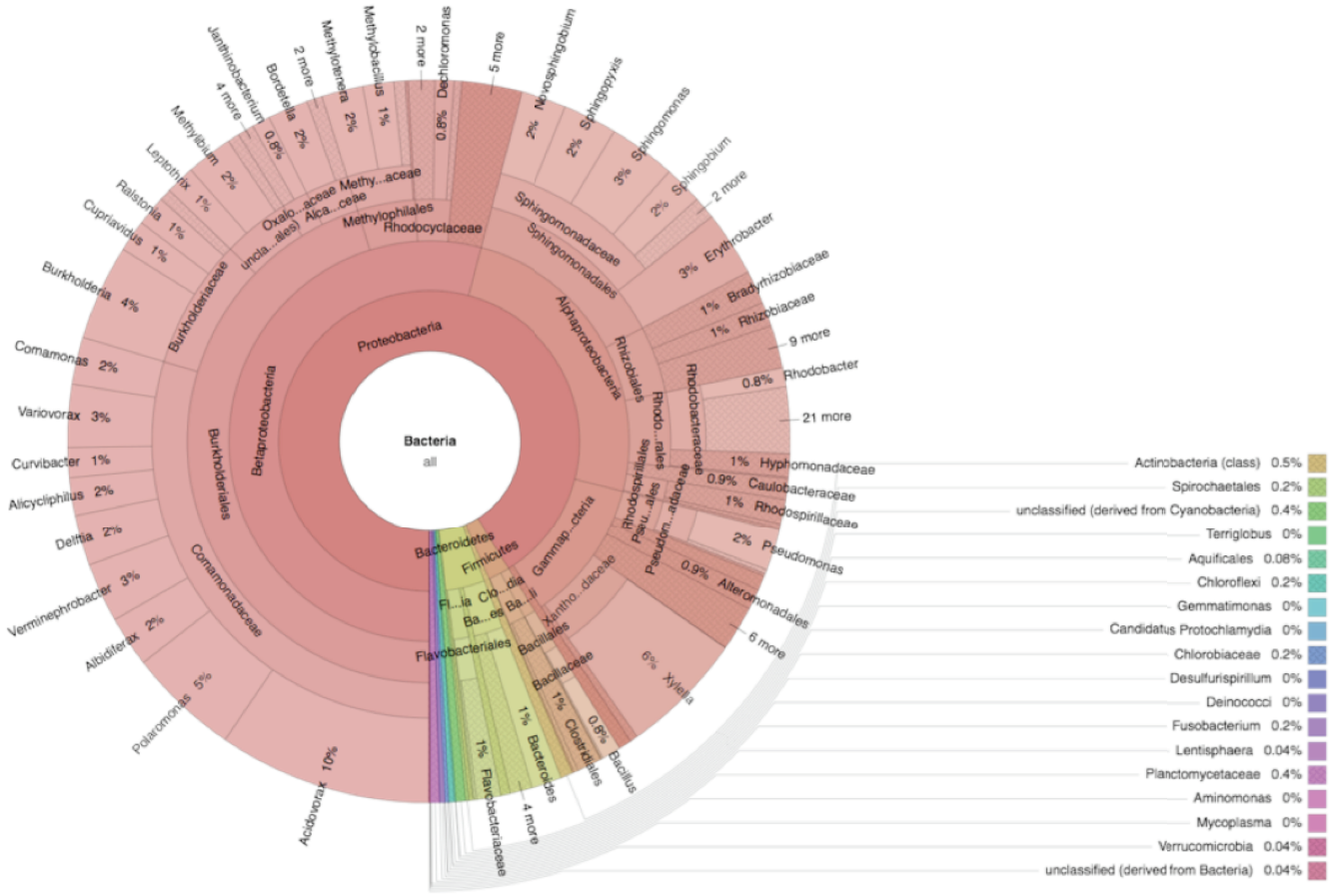

Figure 1. Bacterial diversity identified to genus for a representative *A. obstetricans* tadpole bucal swab (control, S3). See supplement for bacterial diversity figures from three control mesocosms and three probiotic (*F. johnsoniae*-treated) mesocosms representing baseline and altered bacterial assemblages at the phylum level**.**

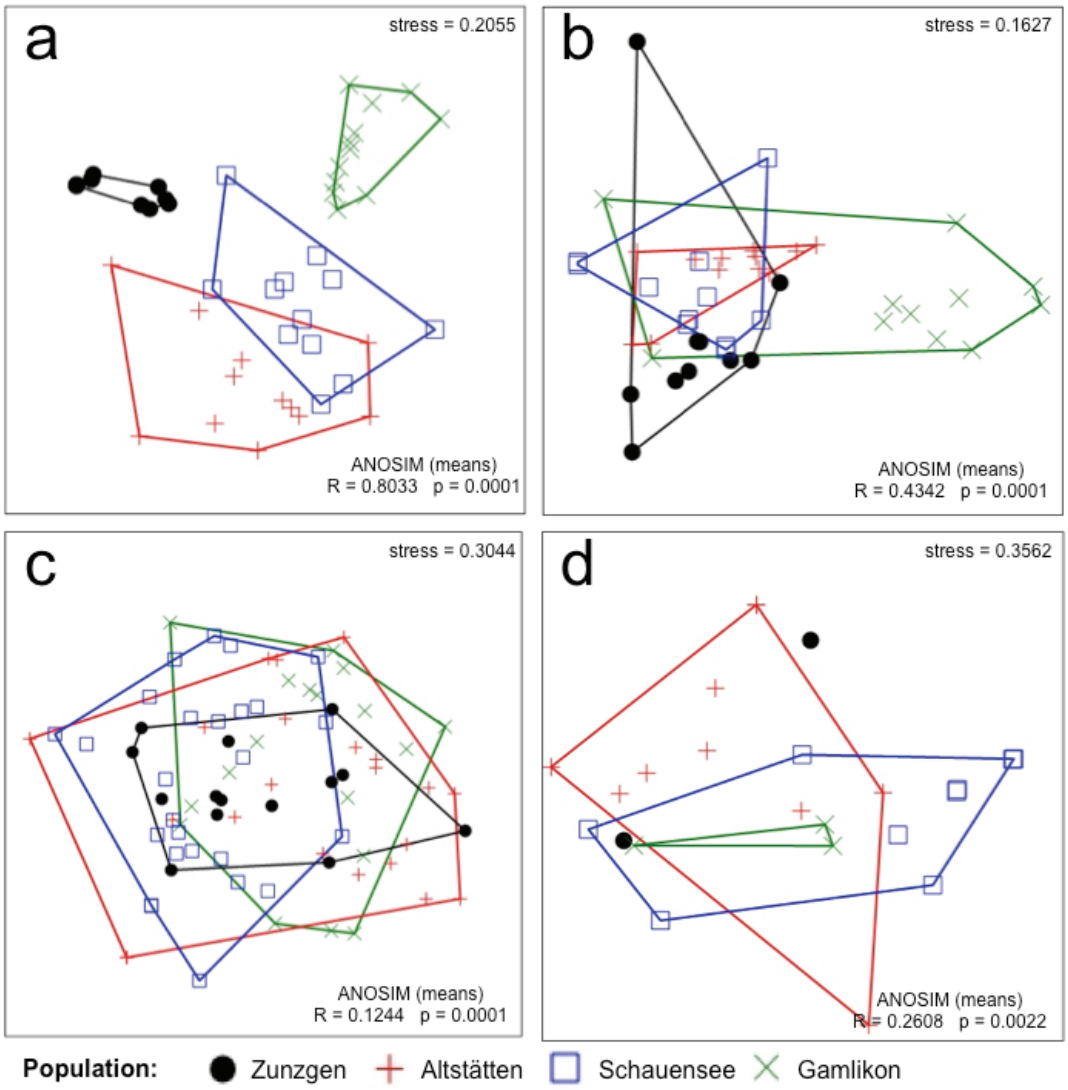

Figure 2. Non-metric multidimensional scaling plots of microbial communities on *A. obstetricans* tadpoles across development and probiotic treatment. The ordination distances are based on a Bray–Curtis dissimilarity matrix for T-RFLP profiles of bacterial 16S rRNA gene amplicons digested with the Hae3 enzyme (a-c) or the fungal ITS gene amplicons digested with Msp1 (d). Each point represents the pooled microbial community on individuals in a tub. Each symbol indicates the population of origin. The distance between points reflects differences in composition. On the basis of ANOSIM (Analysis of Similarity), microbial composition is significantly different for each of the four populations. (a) Plot showing population differences in tadpole buccal cavity bacterial communities upon collection from the wild. (b) Plot showing convergence of tadpole buccal cavity bacterial communities by population one week post-treatment with probiotics. (c) Plot showing population overlap in toadlet surface skin bacterial communities upon metamorphosis. (d) Plot showing tadpole buccal cavity fungal communities from four populations upon collection.

Table 1. Bacterial communities on *A. obstetricans* tested for differences among populations, treatments, and their interaction with 2-way NPMANOVA. In total, 70 bacterial phylotypes were found by T-RFLP using the enzyme Hae3 and 95 phylotypes using Msp1.

Hae3 enzyme		Pre-treatment			Post-treatment			Metamorph Stage 46	
Factor	d.f.	F	*p*	d.f.	F	*p*	d.f.	F	*p*
POPULATION	3	14.792	**0.0001**	3	6.5959	**0.0001**	3	2.421	**0.0001**
TREATMENT	2	0.51318	0.6841	2	0.35331	**0.008**	2	1.1389	0.0682
POP x TRT	6	-1.2655	0.9102	6	0.2214	**0.0004**	6	-2.022	0.2015
Residual	34			33			68		
Total	45			44			79		

Msp1 enzyme		Pre-treatment			Post-treatment			Metamorph Stage 46	
Factor	d.f.	F	*p*	d.f.	F	*p*	d.f.	F	*p*
POPULATION	3	5.9411	**0.0001**	3	3.3478	**0.0001**	3	2.042	**0.0001**
TREATMENT	2	0.52235	0.7574	2	1.1032	**0.0346**	2	0.56256	0.5298
POP x TRT	6	-1.1924	0.881	6	-2.0644	0.138	6	-1.8581	0.1033
Residual	34			45			40		
Total	45			56			51		

Table 2. Fungal communities on *A. obstetricans* tadpoles sampled in the field pre-treatment showed 7 phylotypes when examined by T-RFLP using the enzyme Hae3, and 13 phylotypes when using the enzyme Msp1. The four populations are significantly different by NPMANOVA and ANOSIM analyses.

Enzyme	1-way NPMANOVA			ANOSIM	
	d.f.	F	*p*	R	*p*
Hae3	14	1.838	0.054	0.2547	**0.0088**
Msp1	20	2.445	**0.0079**	0.86438	**0.0001**

Core microbiome through development

Tadpoles hosted a total of 51 phylotypes and metamorphs a total of 70 phylotypes based on T-RFs detected by the Msp1 enzyme (Table S3). Similarly, a greater phylotype richness was detected on metamorphs than on tadpoles by the Hae3 enzyme (Figs. 3, 4a). For an average tub of individuals, a core microbiome, including an average of 3.13 phylotypes is shared by *A. obstetricans* across metamorphosis for an average tub of individuals as visualized by the Venn Diagram (Fig. 4b). An average of 13.6 and 9.53 unique phylotypes are present uniquely among tadpoles and toadlets, respectively. Fluctuations in microbiota over time among individuals of the four populations are illustrated by the phylotype taxa present at each stage (Fig. 3). For instance, post-treatment, most taxa formerly present in the 200-300 bp region are suppressed only to be present once more at the metamorph stage which hosts the greatest number of taxa. There are no T-RF 126 bp peaks in the tadpoles but this T-RF is present in all four populations of the toadlets. The majority of taxa were detected at all three stages though not necessarily in all the same populations at each stage. More than half of the phylotypes (45/70 or 62%) were present in at least two sampling points and 31% (22/70) of phylotypes are shared at all three time points. The number of phylotypes unique to a specific time point was higher in metamorphs (21/70 or 31%) than in tadpoles (1/70 or 0.01%) or post-treatment tadpoles (3/70 or 0.04%).

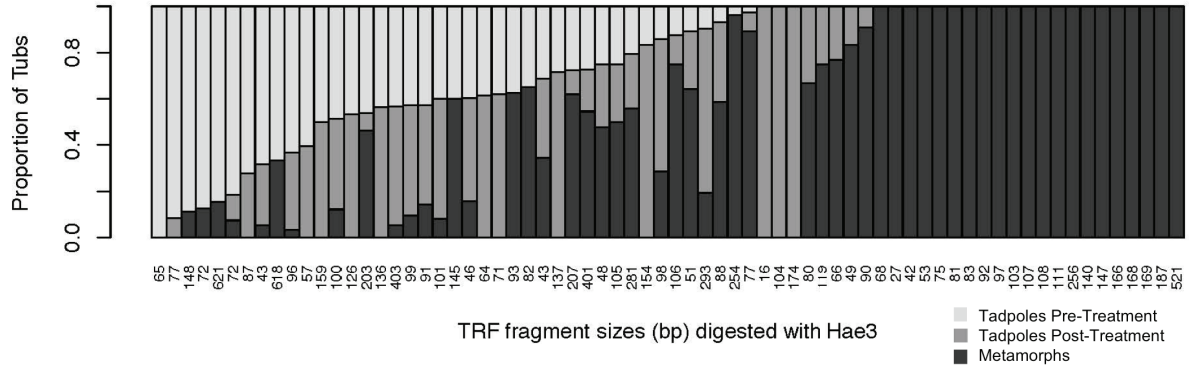

Figure 3. Relative abundance of shared bacterial phylotypes on tadpoles and recent metamorphs. Different phylotypes are represented here by T-RF digests (n=70) of the 16s rRNA gene with Hae3 enzyme. The digest fragment size is along the x-axis. The light, medium, and dark grey bars represent the proportion of tubs sharing a given phylotype (fragment) at each of three sampling points (tadpoles pre-treatment, tadpoles post-treatment, and metamorphosis).

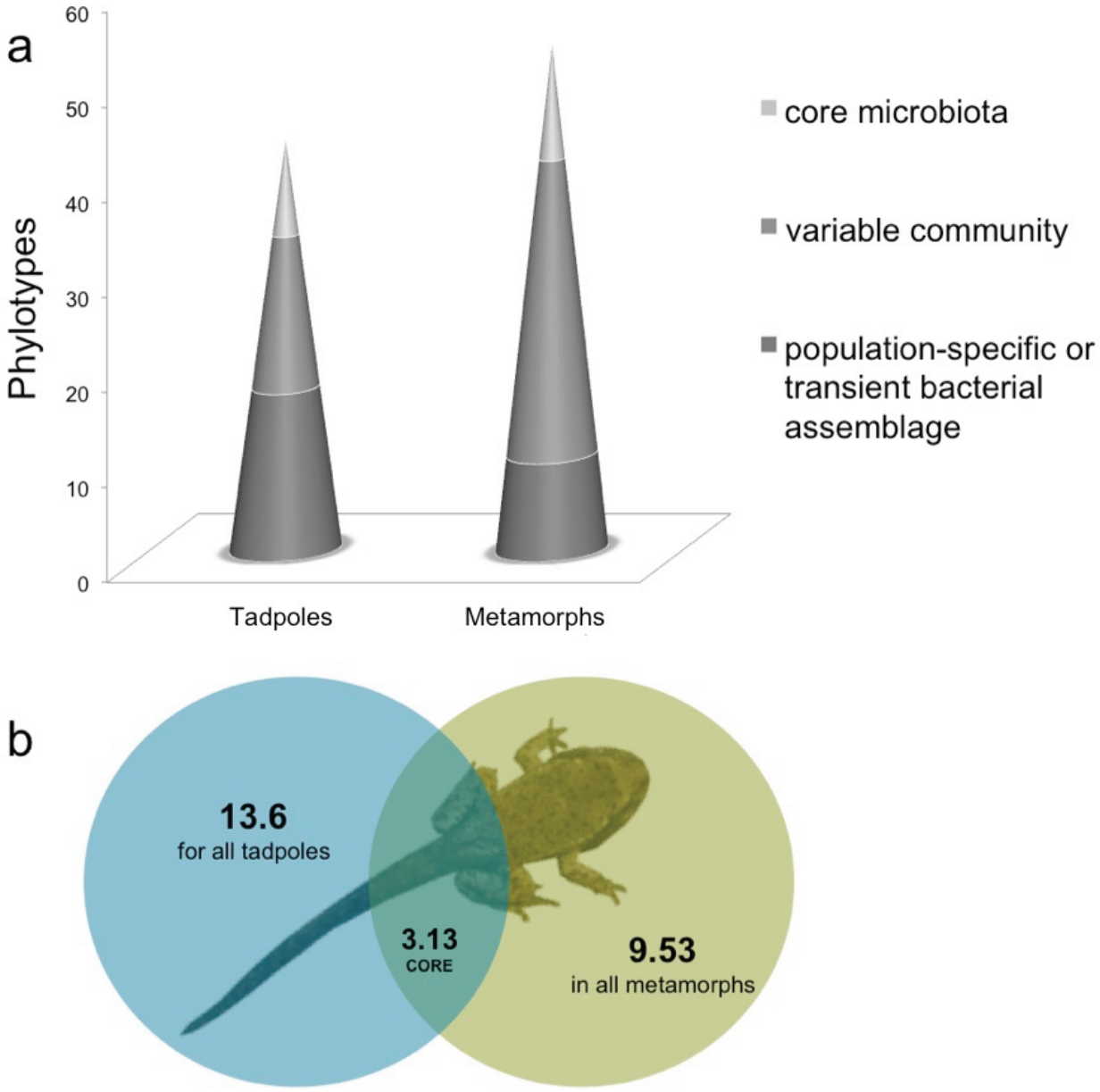

Figure 4. Bacterial phylotypes from *A. obstetricans* shared among developmental stages across metamorphosis and among different populations. The cones (a) divide the raw number of bacterial phylotypes into "core", "variable", and "population-specific" phylotypes present at larval and metamorph stages. The Venn diagram (b) shows the average number of phylotypes (T-RF Hae3 digests) unique to larval and metamorph stages as well as the shared (core) between present at both stages.

Effects of probiotic treatment on bacterial diversity and function

Treatment of tadpoles with the probiotic *F. johnsoniae*, significantly affected the bacterial diversity and functional diversity of the bacterial communities within 1 week post-treatment. Proteobacteria and Firmicutes, two of the most abundant phyla (Figs. 1, S3) showed significantly reduced abundance after probiotic application (Fig. 5). Proteobacteria represented 85-91% of the sequences from control mesocosms and 70-85% of sequences from probiotic mesocosms (Fig. S3). The probiotic itself did not significantly increase in abundance and was detectable on tadpoles before probiotic augmentation (Figs.1, 5).

Functional diversity of the bacterial communities was reduced after application of the probiotic. Principle coordinates analysis of functional gene tags from bacterial assemblages showed a significant shift after probiotic application (Fig. 6a). A heatmap indicates the functional genes such as those involved in iron acquisition and metabolism, and cell division and cell cycle that were most affected by treatment (Fig. S2). The abundance of each unique KEGG pathway was calculated for bacterial tags (393 ECs detected) from tadpoles in control and in *F. johnsoniae* probiotic treatments. Controls contained 71.8% unique functions, and probiotics had 11.7% unique functions. Of the 16.5% of shared ECs, they were significantly more abundant in the control treatment (paired t-test, $t_{64} = 5.079$, $p < 0.001$).

Effects of probiotic treatments on microbial community structure

Probiotic treatments of tadpoles with *P. fluorescens* and *F. johnsoniae* significantly affected bacterial community structure (Fig. 6b). The treatment effects analyzed by NPMANOVA are indicated in Table 1, as well as significant interactions between treatment and population. Analysis by ANOSIM (Table S4) showed similar effects of probiotics on bacterial community structure and also suggested that the effects of treatment may persist through metamorphosis. There was insufficient sample size available to test the impact of probiotics on fungal communities.

Examination of shotgun metagenomes of a subsample of 3 control and 3 *F. johnsoniae* treated mesocosms provided taxonomic information. The most abundant genus in our control tadpole skin samples was *Acidovorax* in the Comamonadaceae comprising up to 10% of sequences (Fig. 1). However, after treatment with *F. johnsoniaea*, *Xylella* in the Xanthomonadaceae became dominant on tadpoles from Schauensee (34–42% of sequences) while *Acidovorax* remained dominant on the tadpoles from Altstätten (8% of sequences).

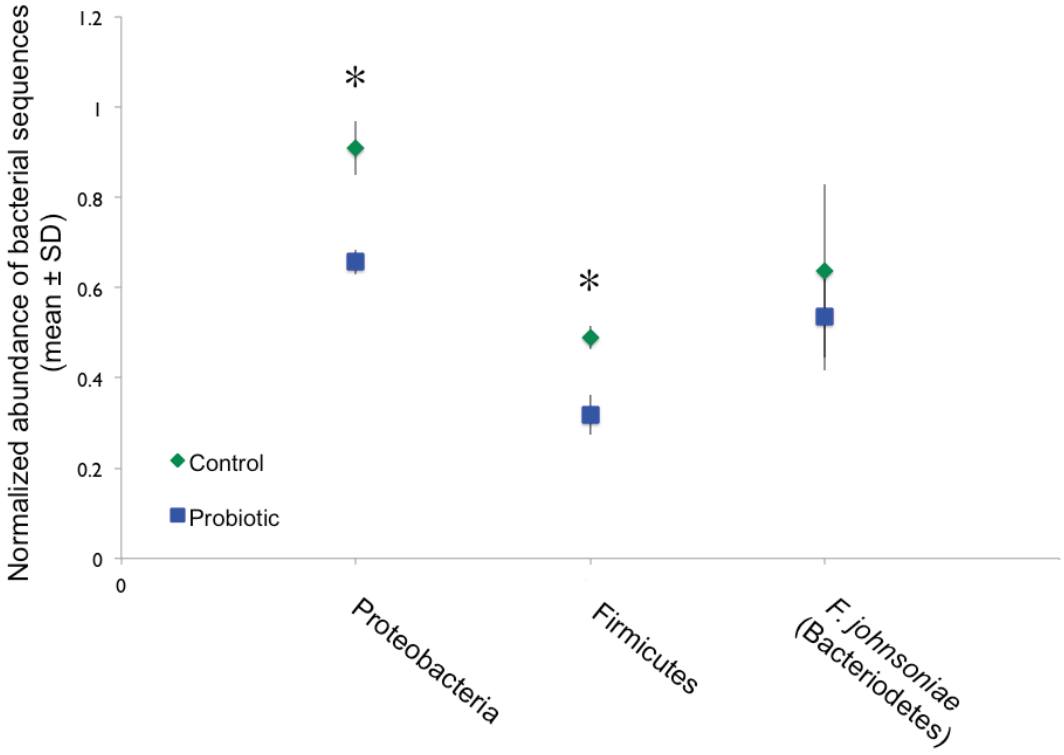

Figure 5. Abundance of bacterial groups on tadpoles treated with a probiotic (*F. johnsoniae*) and controls one week after treatment. Shotgun metagenomes were analyzed in MG-RAST. The * indicates a significant difference between treatment groups.

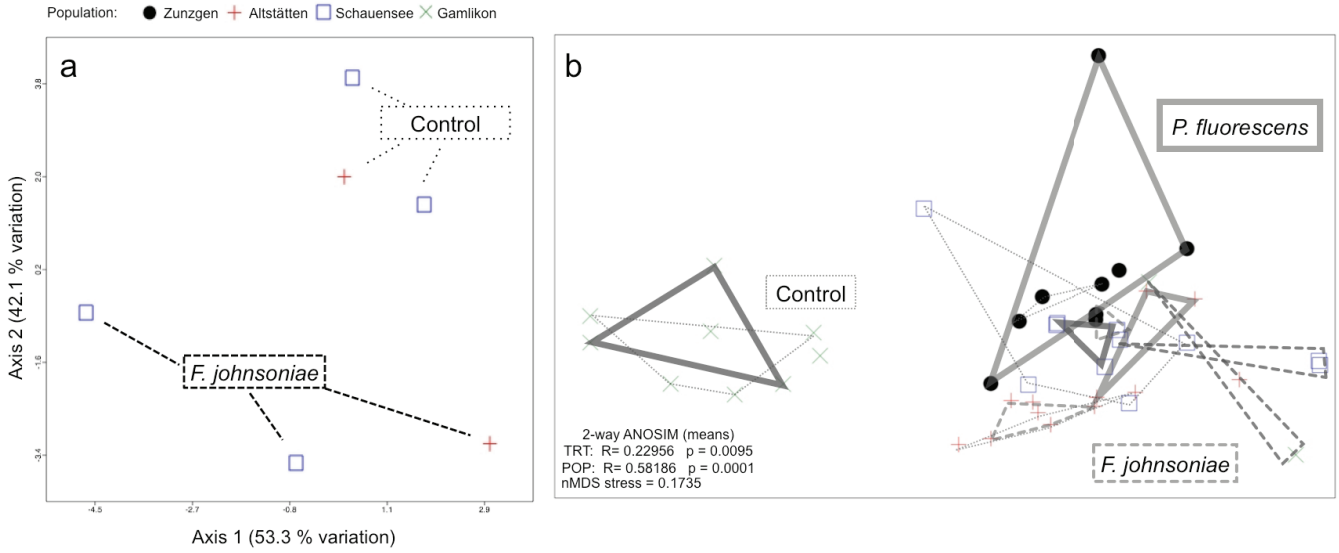

Figure 6. (a) Principle coordinates analysis plot of functional gene tags from bacterial assemblages swabbed from *A. obstetricans* tadpoles one week after treatment in control mesocosms, or mesocosms treated with the probiotic *F. johnsoniae*. (b) An nMDS plot of tadpole bacterial communities one week after treatment, highlighting the interaction of population (symbols by legend) and treatment (convex hulls) for the control (dotted lines), *F. johnsoniae* (dashed lines) and *P. fluorescens* (solid lines) treatment groups. T-RFs from 16S rRNA gene amplicons digested with Hae3 enzyme calculated on relative abundance of peak height, using Bray-Curtis similarities for the ordination.

Skin peptide interactions with microbiota

Of the toads expressing peptides, matching T-RFLP data on bacterial community structure was available from 23 toads. Pairwise correlations between bacterial phylotype (Msp1-digested T-RF) relative intensities and peptide relative intensities yielded significant positive associations (Fig. 7). Alyteserin-2c, previously shown to be a broad-spectrum antimicrobial did not correlate significantly (negatively or positively) with any bacterial phylotype. Of the 46 skin peptides, 14 were positively correlated with 17 bacterial fragments (Fig. 7). A canonical correspondence analysis was used to assess phylotype variation as a function of the 14 explanatory peptides (Fig. S4). Together both axes (22.3% on axis 1 and 17.74% on axis 2) explain 40% of the variation in bacterial community profiles. There is clustering of the Zunzgen population in the lower right gradient with multiple peptides depicting strong positive correlations but not correlated with the other populations. Alyteserin-2c, (1065 *m/z*) and peptide 1671 *m/z* correlate with clustering in the Altstätten and Gamlikon populations. Similarly, peptides 1320 *m/z* and 1244 *m/z* point towards Schauensee and Gamlikon, respectively (Fig. S4).

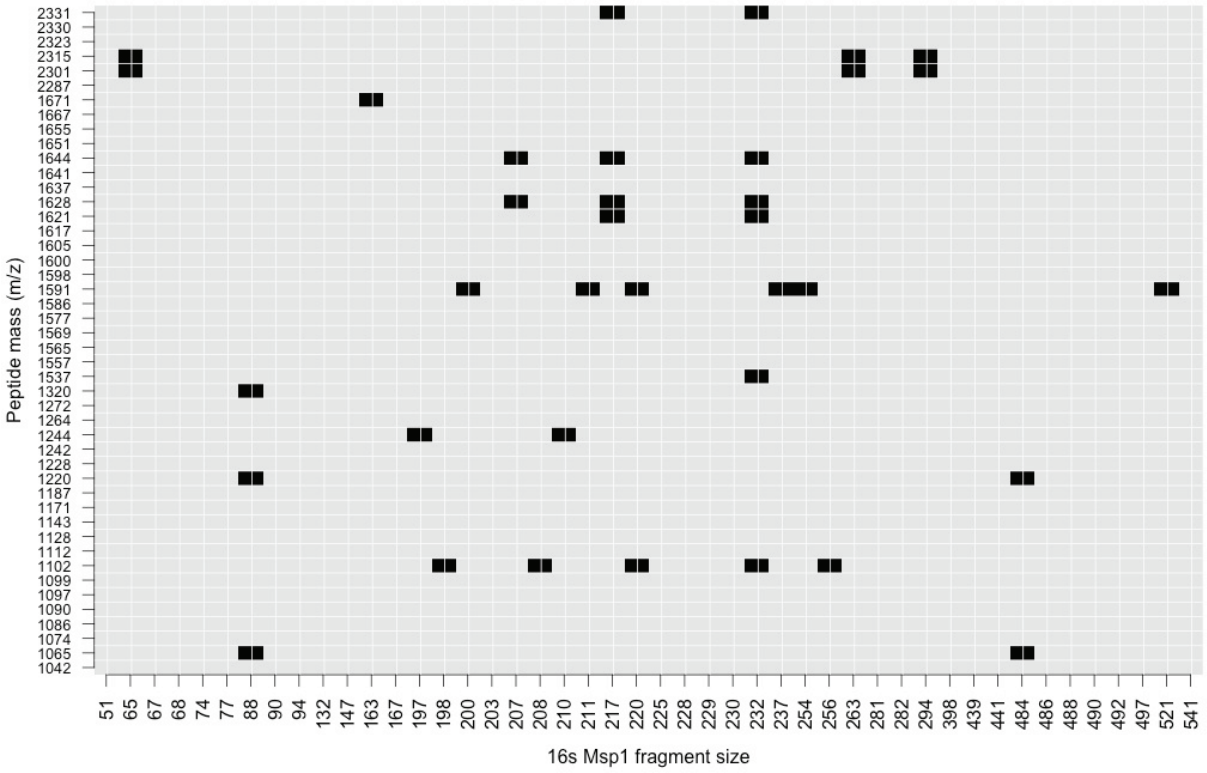

Figure 7. The grid represents all positive pairwise correlations (shaded black) between bacterial T-RFs (digested with Msp1) and peptides detected by MALDI-TOF mass spectrometry and sampled from *A. obstetricans* individuals at metamorphosis.

DISCUSSION

Factors underlying the diversity and stability of host microbiota including the contributions of environment and host immunity are not well understood (Lozupone *et al.,* 2012). We demonstrate that the trajectory of the amphibian skin microbiota after probiotic treatment and through development is strongly influenced by the initial microbial community. Further, the initial host microbiota sampled early in life-history was dependent on pond of origin and is either regulated by the environmental conditions in the pond, or the host genotype and regulatory mechanisms such as oligosaccharides, defense peptides, or other immune defenses of tadpoles and embryos.

The use of probiotics as a remedy or prophylactic for disease is increasing in human and veterinary medicine, in agriculture, and in wildlife conservation (Balcázar *et al.*, 2006; Boyle *et al.*, 2006; Hancock *et al.*, 2012; Preidis & Versalovic, 2009). For amphibians threatened by the emerging chytrid fungus (Fisher *et al.,* 2009; Fisher *et al.,* 2012), probiotic therapy and bioaugmentation strategies are a great hope for conservation. However, few studies have examined whether the established microbial community influences the response of the microbiota to probiotic treatment. Our results suggest that bacterial therapy is not likely to deliver disease prevention equivalently within a host species unless the established microbiota is first reduced or unless treatment is applied at early developmental stages such that the trajectory toward the desired protective and stable-state microbiota is controlled.

Midwife toads harbored population-specific microbiota in their natural habitat, and population differences persisted through time, microbial disturbance by probiotics, and the host physiological and microbial habitat changes associated with metamorphosis. This persistence of distinct microbiota among populations occurred despite housing tadpoles in standardized mesocosm conditions after capture. Our data suggest that host factors, specifically innate immunity, contribute to host establishment and possibly promote microbial persistence. Similarly, symbiosis is maintained in the epithelia of basal animals such as hydra through Toll-like receptors and induction of antimicrobial peptides that help filter out undesirable colonizers (Fraune & Bosch, 2010). Although we cannot exclude the possibility that population-level differences arise by differences in host genotype, or environmental differences from the pond-of-origin, we propose skin peptide defenses as a mechanism contributing to the maintenance of a population-level microbial signature. To our knowledge, this is the first account that chronicles an individual amphibian's microbiota through time and shows a core microbiota shared across temporal (life history) and spatial (population) distance.

Core microbiome among populations and through development

The concept of a core microbiota was first introduced in humans (Sekelja *et al.*, 2012; Turnbaugh *et al.*, 2007) and more recently in sponges (Schmitt *et al.,* 2012) and zebrafish across domesticated and wild-caught hosts from several populations (Roeselers *et al.,* 2011). Consistent with these hosts is our finding that only a small core microbiome of commonly shared and abundant taxa is present among *A. obstetricans* across lifestages and among populations, with the majority of taxa highly population-specific (Schmitt *et al.,* 2012). While these core phylotypes may be critical in maintaining the extended phenotype and amphibian health, there may also be a functional core (Hamady & Knight, 2009; Shade & Handelsman, 2012) that does not require specific symbionts, but rather communities that perform a required function. Identifying specific strains through high-throughput sequencing of 16s rRNA genes and shotgun metagenomics provides deeper insight on the composition, structure, and functional roles of the amphibian skin microbiome (Preidis & Versalovic, 2009). It is possible that the core microbiota provides essential pathogen defense function, or helps stabilize a community incorporating rare members with essential defense functions such as antibiotic secretions. Several antibiotics have been described from cultured amphibian skin isolates that appear to be involved in defense against amphibian chytridiomycosis (Bletz *et al.*, 2013; Brucker *et al.*, 2008a; Brucker *et al.*, 2008b; Harris *et al.*, 2009c). In the shotgun metagenomics approach in this study, we detected these previously cultured microbes thought to be critical components of host defense in other species living in terrestrial environments. However, easily cultivated groups such as *Pseudomonas*, *Flavobacterium*, and *Janthinobacterium* occurred at low abundance (Fig. 1). We also showed that microbial composition changed after treatment of tadpoles with *F. johnsoniae*, but the type of change depended on host population and the initial microbial community.

Overall phylogenetic and functional diversity was reduced following probiotic treatment. This may indicate an extended phenotype response to microbial disturbance with dominant taxa occupying all niches in defense from a potential invader. Several phylotypes that went undetected following treatment re-emerged and were present in all four populations at metamorphosis. These rare phylotypes may become more critical to the host at metamorphosis. Approximately three core bacterial phylotypes were shared between the tadpole and metamorph stage, with a large shift in the microbiota as the host adapted to a terrestrial environment. This shift was influenced by the initial microbiota present, and population-level distinction

observed at the tadpole stage was maintained after metamorphosis. Despite this variation among host populations in the microbiota, it is possible that different combinations of species fulfill similar functional roles as demonstrated in human gut microbiota (Turnbaugh *et al.,* 2007).

Acquisition and maintenance of the skin microbiome

Assembly of the host skin microbiome likely begins at first-contact of the sterile egg to the rich microbial environment. Vertical transmission of microbiota as well as colonization from environmental sources are likely to occur even in species that do not show parental care after egg deposition (Walke *et al.,* 2011). By applying innate immune factors such as antimicrobial peptides to eggs, adult amphibians may be able to reduce infection by pathogens and filter out undesirable colonizers. Eggs of the tropical glass frog, *Hyalinobatrachium colymbiphyllum,* appear to be protected by antifungal defense peptides applied by adults (Walke *et al.,* 2011). By carrying the eggs against the dorsal skin, adult male midwife toads may provide a similar protective function, and influence the microbiota initially acquired by tadpoles as they emerge from the eggs.

After hatching into the aquatic environment, the skin microbiota may quickly establish and be maintained by host factors including mucosal defense peptides (Conlon *et al.,* 2009) or mucin-type glycoproteins with life-stage or population specific oligosaccharide chains (Delplace & Maes, 2002; Varki, 1993). We found positive correlations between several host peptides and bacteria, suggestive of an interaction and co-evolution between symbiotic bacteria and host innate defenses. Yet, rather than simply an induced general defense against pathogens, antimicrobial peptides may primarily function in stabilizing symbiotic microbial communities. The stability of the skin microbiome of amphibians without antimicrobial peptide defenses (Conlon, 2011a) has not been comparatively investigated. We thus propose that defensive skin peptides have a *pro*microbial function that is linked with immune function, and actively maintains or enhances the symbiotic and protective microbiota of host amphibians.

Adding weight to the hypothesis that skin defense peptides may be critical in structuring the amphibian skin microbiome (Conlon, 2011b), Rollins-Smith *et al.,* (2002) showed that *Aeromonas hydrophila*, a common resident on amphibian skin and also an opportunistic pathogen, could tolerate high levels of host antimicrobial peptides. *Pseudomonas mirabilis* and *Serratia liquefaciens* were also found to be resistant to antimicrobial peptides from several host frog species (Schadich & Cole,

2009). The ability of extracellular products of *A. hydrophila* to inhibit amphibian antimicrobial peptides indicates a co-evolutionary relationship between host and symbiont (Schadich & Cole, 2009). Antimicrobial peptides can act synergistically against pathogens (Hancock *et al.*, 2012; Nizet *et al.*, 2001; Vanhoye *et al.*, 2003; Yan & Hancock, 2001), and can act in synergy with bacterial metabolites (Myers *et al.*, 2012). Conversely, the microbiota also play a role in modulating innate immune defenses (Hancock *et al.*, 2012; Ryu *et al.*, 2010; Zasloff, 1992) and the associations we found here between host peptides and bacterial community are likely the outcome of this dynamic interaction.

Implications for amphibian disease susceptibility

The function of host microbiome in immune defense is at the frontier of applications in human medicine (Stecher *et al.*, 2010) and wildlife conservation (Harris *et al.,* 2009b; Lam *et al.,* 2010). Amphibian species differ in resistance to chytridiomycosis (Harris *et al.*, 2009c; Kilpatrick *et al.*, 2010; Woodhams *et al.*, 2006). Similarly, populations of *A. obstetricans* also exhibit differential survival and tolerance of *Bd* (Bosch *et al.*, 2001; Rosa *et al.*, 2012; Tobler & Schmidt, 2010a). Against the backdrop of an emerging disease, knowledge of antifungal components of the amphibian microbiome (Becker *et al.*, 2011; Harris *et al.*, 2009b; Woodhams *et al.*, 2007b) and our finding that amphibians have population-specific bacterial communities on their skin suggests a potential link between microbiota and within-species disease risk. The ability of probiotics or bioaugmentation strategies to reduce disease risk (Bletz *et al.*, 2013; Woodhams *et al.*, 2011) may depend on initial microbial conditions. Customizing treatments tailored to an individual's initial microbiota may require strategies for specific host populations, habitats, or life stages, or methods to standardize initial conditions such that the trajectory of the microbial community can be controlled. However, hosts may differ in their capacity to stabilize a protective skin microbiome, and stabilizing factors such as defensive peptides may provide effective prebiotics as an alternative to microbial augmentation strategies.

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SUPPLEMENTARY INFORMATION

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EXTENDED EXPERIMENTAL DESIGN

All midwife toad, *Alytes obstetricans*, tadpoles in this study were collected in the wild from man-made ponds (Table S1), including two dugout PVC-lined ponds featuring a muddy bottom and thick surrounding vegetation. One such pond was found in canton Basel (BS-Z) and the other in canton Zürich (ZU-G). The third pond, in St. Gallen (SG-A) had a rocky bottom and little natural substrate and very densely covered by algae. The last site featured a regularly maintained clear-water fountain on the grounds of a manicured lawn of the Schauensee museum in Kanton Luzern (LU-S). A shrub brush sheltered the pond but otherwise there was no natural vegetation or visible algae (Table S1). This was also the site of reported mass mortalities of young metamorphs in August 2010 (C. Geiger, *pers. comm*.). The importance of population-based susceptibility and survival to *Bd* was shown by Tobler and Schmidt (2010c), where two of the study populations were previously examined. Although no significant difference in survival of infected tadpoles was observed between BS-Z & SG-A, there was an outstanding difference when compared to the third population, Itingen (Tobler and Schmidt 2010). Unfortunately, the 2011 overwintering population from the Itingen pond could not be included in this study due to insufficient sample size.

Quantitative PCR

To determine *Bd* infection loads, the Boyles et al. (2004) real time PCR protocol was employed for the pooled DNA extracts of each tub and *Bd*-specific primers from Microsynth: ITS1 (5'-CCT TGA TAT AAT ACA GTG TGC CAT ATC TC-3') and 5.8s (5'-AGC CAA GAG ATC CGT TGT CAA A-3'). Protocol modifications included: running 50 cycles, repeating the analysis when samples run in duplicate yielded

inconsistent SDs greater than 0.5, and the FastStart Universal Probe Master was acquired from Roche: Chytridprobe, 6-FAM-CGA GTC GAA CAA AAT- MGB (Roche, Basel, Switzerland). All runs were quantified based on zoospore standards acquired from EcoGenics (Zürich, Switzerland, 2010) and stored at -80°C.

T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) is a high throughput, high resolution and community fingerprinting method implemented to monitor bacterial and fungal microbiota communities through time. Despite known biases of PCR, amplification efficiency, and capillary electrophoresis, T-RFLP can reliably detect changes in structure and diversity of a bacterial community with consistent results (Hartmann & Widmer, 2008). Labeled primers for the universal bacterial 16S and fungal ITS universal primer pairs were selected as the community target genes for optimizing thermocycling conditions. The 16S rDNA region was amplified using the primer 27F (PET® labeled) (5'-AGA GTT TGA TCC TGG CTC AG-3') from Applied Biosystems and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3') primer from Microsynth. The multiplex also included an ITS rDNA primer, the forward primer, ITS1 (YAK® labeled) (5'- CC GTA GGT GAA CCT GCG G-3') from Applied Biosystems and the reverse ITS4 primer (5'-TCC TCC GCT TAT TGA TAT GC-3') from Microsynth. A 20 µl Mastermix was prepared to run the PCR reaction for each sample in triplicates under a sterile hood to contain: 2 µl template DNA, 1 µl of each of the four primers (10µM), 1 µl BSA (2ug/µl), 2 µl dNTPs (2mM), 4 µl buffer (5x), 1.2 µl $MgCL_2$ (25mM), 1 µl Taq 1:10 (0.5u/µl), and 6.8 µl nuclease-free PCR water.

Steps for the multiplex PCR runs involved: amplification at an initial denaturation at 95 °C for 5 min, followed by 32 cycles with denaturation for 1 min at 94 °C, annealing for 1.5 min at 52 °C, extension for 2 min at 72 °C, and a final elongation step for 10 min at 72 °C. All samples were run under these conditions except for the individual Stage 46 swabs that did not yield sufficient DNA to run a multiplex PCR; for these, a singleplex 16S PCR program was run with the slight modification of 95°C at denaturation. Triplicates were pooled and bacterial and fungal amplification was confirmed via gel electrophoresis on a 1% agarose gel stained with GelRed® (visualized at 302 nm). Pseudoterminal single-stranded fragments were reduced by treating the digestion with 10 units of mung bean nuclease (New England BioLabs, USA) with 12 µl of 10x reaction added to the PCR product. After digestion of single-stranded DNA for 2 hours at 30 °C, the reaction was disrupted with 0.01% SDS. PCR products and mung bean fragments were purified following the GenElute PCR Clean-Up Kit (Sigma-Aldrich) protocol with the modification in the final step elution with 33 µl to yield a final volume of 30 µl.

Purified DNA product was quantified by a Nanodrop spectrophotometer (absorption at 260nm). If necessary, samples were diluted with MiliQ water to a final DNA concentration of 50 ng/µl and resuspsended to 200-500ng before restriction digestion. Three enzymes (Hha1, Hae3, and Msp1) were tested in preliminary trials at different incubation times and digestion volumes and optimized for selected cultured bacterial and fungal isolates (from *Alytes obstetricans*) and in some microbial community DNA samples. Two enzymes, Msp1 and Hae3 (Fermentas) were added to the 20 µl reaction by adding 1.5 units of each enzyme diluted in 2 µl of 10x Tango Buffer or 10x FastDigest, respectively. After centrifuging, the plate was incubated at 37°C for 3 hours. A 17.8 µl HiDi-Formamide (Applied Biosystems) and 0.2 µl GeneScan 500 LIZ size standard (Applied Biosystems) was added to each sample and denatured for 20 minutes at 80°C. Fragments were sized with a 36 cm multicapillary ABI 3730 DNA Analyzer (Applied Biosystems) and processed with the GeneMapper v 3.7 (Applied Biosystems). T-RF sizes and intensities were determined with the AFLP and Local Southern size calling options. Automated peak binning was generated with the following parameters: polynomial degree of 4, a window size of 13, and a minimum width at half maximum of 2. The analysis was restricted to the 50-620 bp range for both enzymes in order to exclude artifacts and primer dimers and only peak intensities greater than 200 RFU (relative fluorescence units). A further reduced range (80-600 bp) of fragment tests were also run for impact on overall results. Individual spectra were examined manually for peak calling and binning and edited in Microsoft Excel. Samples with less than three peaks were excluded from further analysis. Datasets were standardized by calculating the total peak area (or intensity) for every sample and dividing the area (or intensity) of each peak by the total area (or intensity) in order to obtain relative peak areas and relative heights respectively for every sample.

T-RFLP Enzyme Efficiency

Of the three enzymes tested on bacterial and fungal communities of amphibian skin, Msp1 and Hae3 provided distinct peaks and the most T-RFs. Both enzymes yielded similar patterns of bacterial diversity, with Msp1 providing a higher resolution compared to Hae3. Total unique bacterial T-RF taxa for each hydrolysis was n=67 (Hae3) and n=95 (Msp1) fragments for all tadpoles and toadlets sampled over three timepoints from the 48 tubs in this study (Table S2). Although Msp1 was successful in yielding more T-RFs, there was a higher failure rate with Msp1-digested samples and thus the overall sample size is often reduced for certain tests where it is more reliable to compare with the more complete sample size in the Hae3 data set. Similar patterns were found for relative peak height as for relative peak area.

Microbiota Analysis by Shotgun Metagenomics

Six libraries were prepared and sequenced using manufacturer-supplied protocols (Meyer *et al.,* 2008) and reagents (Roche GS-FLX XLR70 Titanium chemistry), as follows. 500 nanograms of DNA was sheared to an average size of 500 bp. Library-specific MID Adaptors were ligated, and the correct products were selected using 454 library immobilization beads, in an automated fashion using the SPRI-TE™ Nucleic Acid Extractor (Beckman Coulter). The single-stranded DNA libraries obtained were quantified using the single cuvette TBS 380 Fluorimeter (Turner Biosystems). 1E7 molecules/□l – diluted libraries were pooled in equimolar ratio prior emulsion PCR reaction, which was prepared with a ratio of one molecule per DNA capture bead. After amplification, the emulsions were broken and enriched, resulting in a total of 2.85 million beads containing amplified library fragments. The pooled sample was sequenced on a full picotiterplate on a Genome Sequencer FLX Instrument. Sequencing raw data were processed with the GS Run Browser (version 2.5.3) using standard quality filtering and trimming as defined by the default settings. A total of 1,049,935 reads passed the filters with a median length of 280 nucleotides.

Peptide Sampling

Newly metamorphosed toads were sampled for skin peptides in August 2008. Animal care and experimental procedures were approved by the Cantonal Veterinary Office of Zurich and Kanton Basel-Landschaft. Granular skin secretions were induced by administration of norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri) by subcutaneous injection (40 nmoles per g body mass; Rollins-Smith et al. 2002, Woodhams et al. 2006b). After administration of norepinephrine, skin secretions

were collected for 15 min in water and acidified with 1% hydrochloric acid to help prevent proteolytic degradation of the samples. Skin secretions were then partially purified by passing over C-18 Sep-Pak cartridges (Waters Corp., Milford, Massachusetts) activated with methanol, eluted in buffer containing 70% acetonitrile and 0.1% TFA, and spun dry with heat. Dry weight of peptide-enriched samples was measured before analysis by matrix-assisted laser desorption/ionization (MALDI) using an Autoflex I time-of-flight mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a 337nm nitrogen laser. A 20 µl sample solution at a concentration of 1 $mg*ml^{-1}$ peptides was diluted with 20 µl H_2O + 0.1% trifluoroacetic acid, vortexed and 1 µl was spotted on a "Prespotted AnchorChip" target prepared with α-cyano-4-hydroxycinnamic acid as matrix (HCCA, Bruker). Instrument calibration was obtained using signals from the HCCA matrix at m/z 379.09 and a mixture of standard peptides composed of Bradykinin 1-7 (m/z 757.40), angiotensin II (m/z 1046.54), angiotensin I (m/z 1296.69), renin substrate (m/z 1758.93), ACTH clip 18-39 (m/z 2465.20) and Somatostatin 28 (m/z 3147.47) all obtained from the peptide calibration standard mix II (Bruker).

Is survival post-metamorphosis correlated with peptides or *Bd* infection?

Skin peptides were sampled from toadlets at the end of the summer growing season. All individuals at this stage had extremely low zoospore loads from 0 to 9 zoospore equivalents. Thus, deaths at this stage were not due to severe *Bd* infection loads, and survival for 32 days of observation after peptide sampling was not linked to peptide presence or profile (NPMANOVA, $F = 0.5563$, $p = 0.6089$). Neither was survival time in days significantly correlated with the relative intensity of any peptide (Bonferroni adjusted p-value for multiple comparisons = 0.001, Kendall's tau, all $p > 0.001$).

SUPPLEMENT TABLES

Table S1. Pond characteristics for source populations of midwife toads, *A. obstetricans*.

Enzyme	Factor	Pre-treatment tadpoles			Post-treatment tadpoles			Metamorph Stage 46		
		n	R	*p*	n	R	*p*	n	R	*p*
Hae3	Population	46	0.80554	**0.0001**	48	0.58186	**0.001**	57	0.20804	**0.0003**
	Treatment	46	-0.03635	0.6599	48	0.22956	**0.0095**	57	0.1064	**0.0237**
Msp1	Population	46	0.86438	**0.0001**	37	0.38476	**0.0001**	53	2.042	**0.0001**
	Treatment	46	-0.08773	0.8802	37	0.1858	**0.0143**	53	0.56256	0.5298

Table S2. Summary of shotgun metagenomics data obtained from six groups of five *A. obstetricans* tadpoles swabbed across the denticles and body after rinsing. Initial processing including quality control (QC) was performed under metagenome rapid annotation using subsystem technology (MG-RAST; http://http://metagenomics.anl.gov/) where the data have been made publically available.

	Control treatment			**Probiotic treatment**		
MG-RAST ID	4475938.3	4475941.3	4476038.3	4475968.3	4476033.3	4476035.3
Sample name	S1	S3	A8	S4	S6	A4
Base-pair count	62581964	99345546	87805901	51805553	61716866	66453395
Sequence count	159044	233918	207654	122937	152495	168806
Sequence count post QC	106197	158499	137655	81758	101473	114685
Mean sequence length post QC	387	427	426	421	402	388
Identified rRNA features[a]	1452 (1.37 %)	2046 (1.29 %)	2180 (1.58 %)	925 (1.13 %)	1838 (1.81 %)	1857 (1.62 %)
Archaea	44 (0.22 %)	79 (0.25 %)	76 (0.31 %)	45 (0.38 %)	33 (0.21 %)	46 (0.29 %)
Bacteria	2174 (10.64 %)	4159 (13.03 %)	6032 (24.49 %)	1271 (10.62 %)	1514 (9.63 %)	1695 (10.59 %)
Eukaryota	17521 (85.77 %)	26640 (83.49 %)	17716 (71.94 %)	10113 (84.54 %)	13565 (86.30 %)	13736 (85.79 %)
Viruses	602 (2.95 %)	881 (2.76 %)	636 (2.58 %)	450 (3.76 %)	484 (3.08 %)	475 (2.97 %)
Other	87 (0.43 %)	148 (0.46 %)	166 (0.67 %)	84 (0.70 %)	122 (0.78 %)	59 (0.37%)
Total coding sequences (EGTs)[b]	20428 (19.24 %)	31907 (20.13 %)	24626 (17.89 %)	11963 (14.63%)	15718 (15.49 %)	16011 (13.96 %)

a The E value cutoff for SSU rRNA hits for all databases used is 10-10 with a minimum length of 45 bp
b The BLASTX cutoff for environmental gene tags (EGTs) is 1 x 10-5

Table S3. Phylotypes (T-RFs) detected for each enzyme at each stage of the experiment and end-total unique number of T-RFs.

	Hae3	**Msp1**
Tadpoles, pre-treatment	38	51
Tadpoles, post-treatment	36	36
Metamorphs, stage 46	51	70
Total T-RFs	*70*	*95*

Table S4. ANOSIM of bacterial communities on *A. obstetricans* analyzed by T-RFLP.

Enzyme	Factor	Pre-treatment tadpoles			Post-treatment tadpoles			Metamorph Stage 46		
		n	R	*p*	n	R	*p*	n	R	*p*
Hae3	Population	46	0.80554	**0.0001**	48	0.58186	**0.001**	57	0.20804	**0.0003**
	Treatment	46	-0.0364	0.6599	48	0.22956	**0.0095**	57	0.1064	**0.0237**
Msp1	Population	46	0.86438	**0.0001**	37	0.38476	**0.0001**	53	2.042	**0.0001**
	Treatment	46	-0.0877	0.8802	37	0.1858	**0.0143**	53	0.56256	0.5298

SUPPLEMENT FIGURES

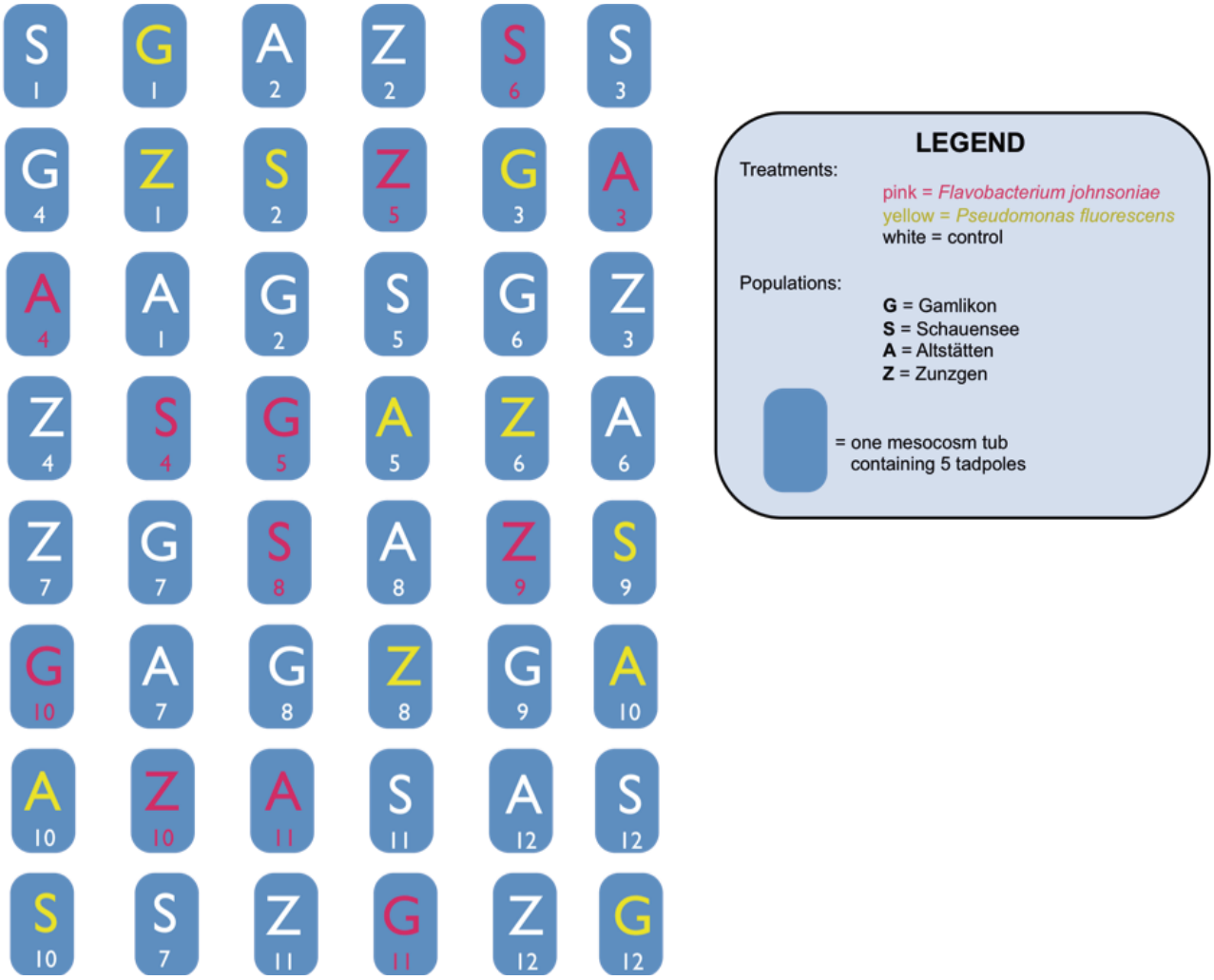

Figure S1. Layout and block design of outdoor mesocosms for *A. obstetricans* tadpoles

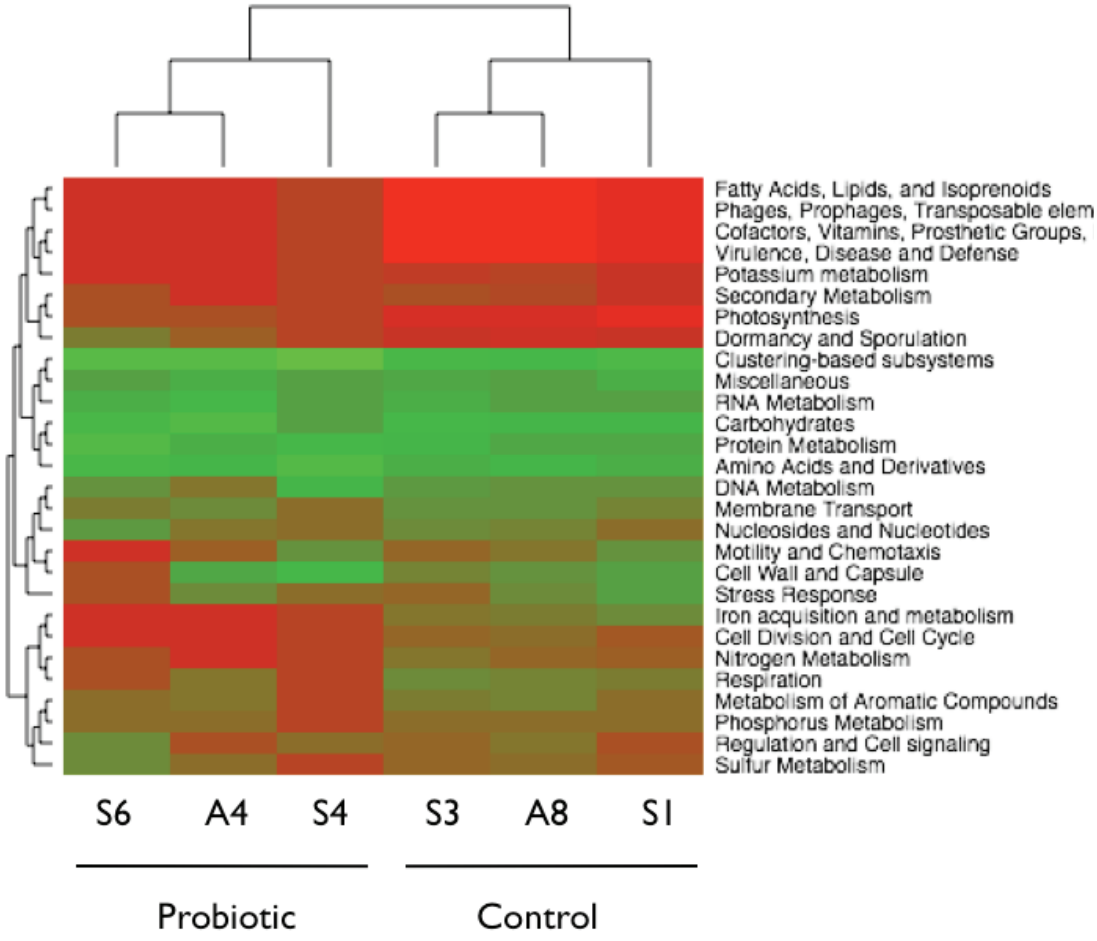

Figure S2. Heat map of functional gene tags from bacterial assemblages swabbed from *A. obstetricans* tadpoles in mesocosms one week after treatment with the probiotic *F. johnsoniae* or control. Sample names and mesocosm identification are described in Table S2.

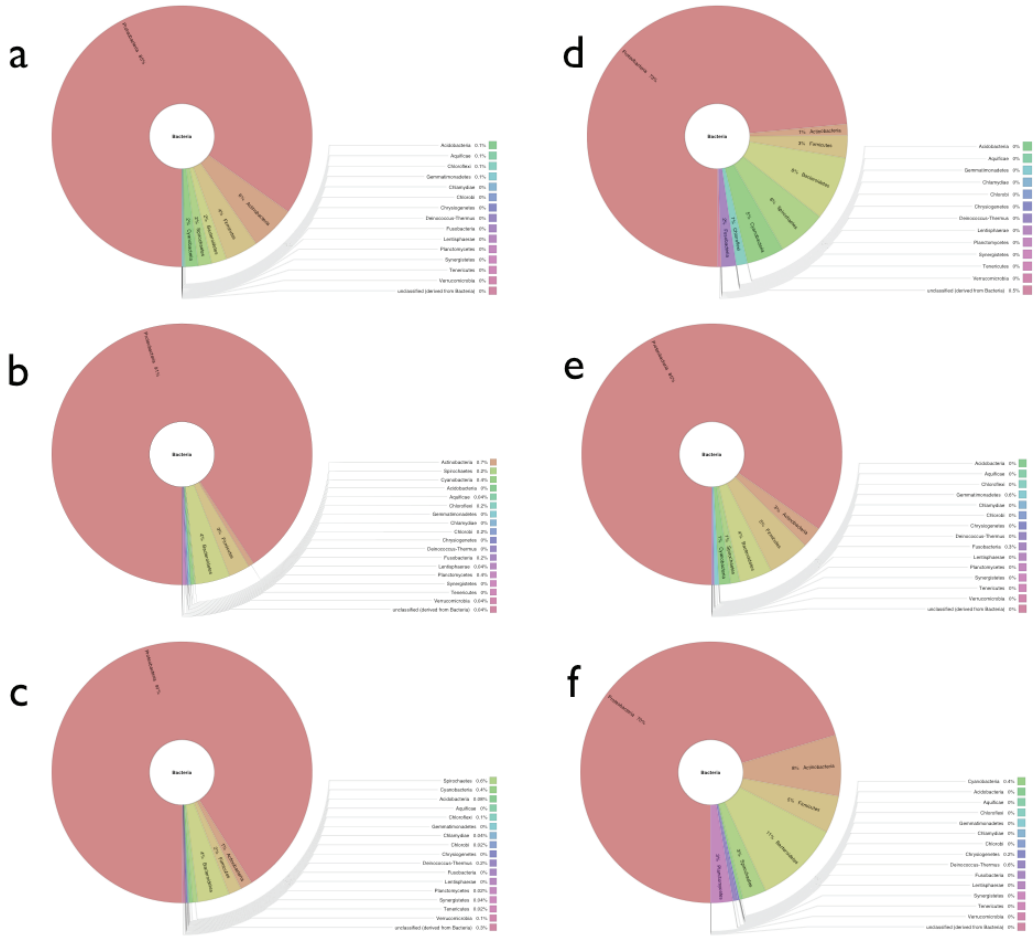

Figure S3. Phylum-level bacterial diversity on larval midwife toads, *A. obstetricans*, from three control (left panels) and three probiotic (right panels; *F. johnsoniae* treated) mesocosms. Sample names correspond to description in Table S2: a) S1, b) S3, c) A8, d) S4, e) S6, f) A4.

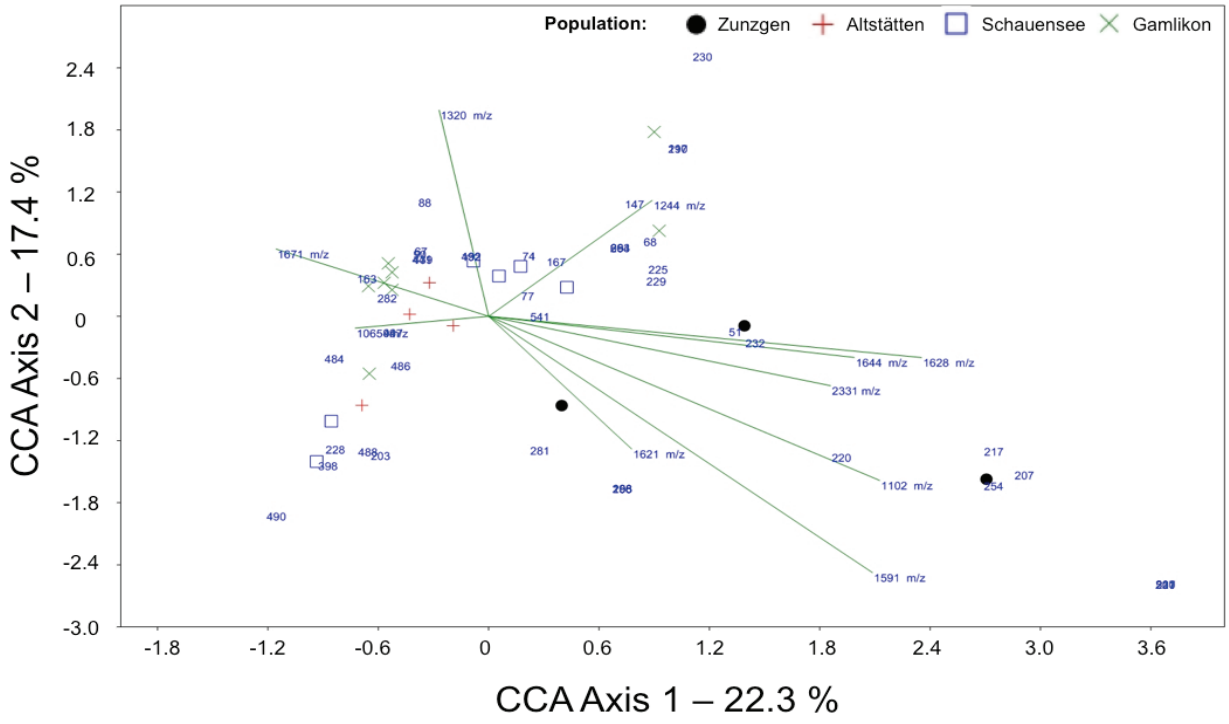

Figure S4. Canonical Correspondence Analysis (CCA) of the bacterial community profiles for *A. obstetricans* toadlets, with mucosal defense peptides as a physiological explanatory variable. The biplot shows the interaction of surface bacteria (n = 46 T-RFs) from host *A. obstetricans* (n = 22 toadlets) and the host's secreted peptides (n = 10 peptides) as explanatory variables. Lines pointing in the same direction indicate positively correlated variables whereas lines at a 90 degree angle depict uncorrelated variables. These include 10 explanatory peptides within a repertoire of 46 secreted peptides present in *A. obstetricans* metamorphs. This does not mean that the other peptides do not contribute to variation in bacterial communities, only that they are less predictive. The predictive peptides were first identified through the individual TRF-AMP pairwise correlations between peptides and bacteria (Fig. 7) to identify potential parameters of variation.

CHAPTER THREE

Symbiont antifungal therapy improves survival through metamorphosis for an endangered amphibian under semi-natural conditions

Leyla R. Davis, Benedikt R. Schmidt, and Douglas C. Woodhams

ABSTRACT

Chytridiomycosis is an emerging fungal disease that is a present threat for amphibian species worldwide and has been met with various strategies for mitigation. Bioaugmentation is one such intervention embraced recently for offering minimal ecosystem invasiveness and the potential of a sustained, cross-generational impact. We tested probiotic therapies in semi-natural outdoor mesocosms with 240 tadpoles of the midwife toad, *Alytes obstetricans*, collected from four populations in Switzerland, naturally infected with *Batrachochytrium dendrobatidis* (*Bd*). Because morbidity in this species occurs upon metamorphosis, tadpoles were treated with symbiotic bacteria isolated from adult toads persisting in one of the study's population. These isolates were selectively screened for antifungal and probiotic properties. After treatment with the symbionts *Pseudomonas fluorescens*, *Flavobacterium johnsoniae*, or water controls, tadpoles were raised through metamorphosis. Infection loads were monitored at multiple time points and survival through metamorphosis to the juvenile stage recorded. Symbiont therapy improved survival compared with the control group; *P. fluorescens* was associated with a significantly increased survivorship and *F. johnsoniae* reduced zoospore loads in two of four populations one week post-treatment. While disease-induced mortality differed among populations, the absence of a significant treatment-by-population interaction indicates that all populations of this species could benefit from a probiotic therapy. A critical goal in the development of management strategies is reproducing laboratory success in the field. Here we report the first documented trial of probiotic therapy of infected larvae under semi-natural conditions. Based on the outcome, we suggest targeting future bacterial therapy research towards early exposure of larvae upon hatching, and repeated probiotic treatments to enhance microbiota critical to amphibian health and improve survival through metamorphosis.

INTRODUCTION

Emerging infectious diseases are on the rise, threatening the loss of biodiversity in various systems (Fisher *et al.*, 2009). Amphibians are currently experiencing one of the most dramatic biodiversity declines across the planet at a pace comparable with mass extinctions (Wake & Vredenburg, 2008). The proximate cause is the emerging fungal disease chytridiomycosis, the "worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon *et al.*, 2005). The etiological agent of this disease is the ubiquitous fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Farrer *et al.*, 2011), a present threat to nearly a third (Stuart *et al.*, 2004) of the roughly 7,000 extant amphibian species across three distinct and diverse clades, including the anurans, caudatans, and gymnophiona (Gower *et al.*, 2013). The time-sensitive and planetary proportions of this lethal disease has cued a burgeoning of interdisciplinary methods to mitigate, prevent, or treat infections, which underscores a need for a global strategy to control *Bd* (Daszak, 2000; Daszak *et al.*, 2004).

A component to developing a mitigation method is targeting a stage critical within a host's life history. An important consideration is the biphasic life cycle in amphibians, adding a layer of complexity to the treatment target. Further, susceptibility to a pathogen has been widely documented to change with development (Johnson *et al.*, 2011), with most die-offs occurring after metamorphosis (Haislip *et al.*, 2011). This narrow window is associated with natural immune suppression function under metamorphosis associated with endogenous production of gluccocorticoids during adaption of the internal organs to a post-metamorphic life (Gray *et al.*, 2009). An important component of this study was to target the infected larvae, a stage where infection prevalence is highest, when capture and treatment *en masse* is most feasible, and when surmounting metamorphosis is critical to long-term survival. To our knowledge, no prior studies have examined the efficacy of bioaugmentation at the larval stage through post-metamorphic survival.

Tadpoles of many species including our focal species, the midwife toad, *Alytes obstetricans*, can be infected without showing clinical signs of disease (Garner *et al.*, 2009). However, upon metamorphosis, sub-lethal and lethal effects (Blaustein *et al.*, 2005; Garner *et al.*, 2009; Parris & Cornelius, 2013) can occur as the fungus spreads from the denticles of a tadpole to the keratinized skin of emerging toadlets (Berger *et al.*, 2005; Berger *et al.*, 1998); a scenario witnessed in Spain during one of the earliest documented mass population declines of chytridiomycosis in the midwife toad (Bosch *et*

al., 2001). Declines in other European populations have been documented (Pasmans *et al.*, 2010) and within Switzerland, nearly half of all *A. obstetricans* populations have gone extinct (Schmidt & Zumbach, 2005). However several populations still persist with *Bd* and the link between *Bd* and recent declines remains unclear (Tobler & Schmidt, 2010). If *Bd* continues to threaten host populations, disease mitigation should aim for the most susceptible life-history stage (Garner *et al.*, 2009) in order to ensure survival of a viable population (Di Minin & Griffiths, 2011; Hels, 2002; Lampo & Giulio, 1998).

The skin surface ecosystem balance plays a central role in regulating host health through dynamic interactions of innate immune defenses and an indigenous microbiota (Rollins-Smith *et al.*, 2011). Harnessing these surface symbionts to enhance innate immune defenses is a commonly practiced strategy referred to as bioaugmentation or probiotic therapy. While widespread use has proved beneficial in a variety of agricultural, veterinary, and medical contexts (Balcázar *et al.*, 2006; Preidis & Versalovic, 2009), emerging findings suggest that probiotic therapies can also be effectively applied against wildlife diseases including chytridiomycosis under controlled conditions (Harris *et al.*, 2009; Vredenburg *et al.*, 2011). Here, we introduce symbiont therapy, exposure to a microbe previously cultured from a healthy host collected from this species and locality and previously identified to play a protective role as a member within the host's microbial community (Davis and Woodhams, *unpublished*).

Probiotic therapy is a particularly promising antifungal treatment for its long-term potential. As *A. obstetricans* is a social amphibian, oftentimes found huddled in groups gathered under crevices and rocks underground, such behaviors may grant opportunity for adult-adult transmission of microbiota. The unique reproductive behavior of midwife toads is for males to string the fertilized egg masses on their legs and care for them until they are developed and carry them to be deposited at the pond surface. Should a probiotic successfully establish within the host microbial community, particularly within the context of our host system, the possibility for vertical and horizontal transmission across and among generations within a population is imminent (Walke *et al.*, 2011). While laboratory trials provide a proof-of-concept for probiotic therapy, the context-dependency of microbiota on temperature conditions alone (Daskin & Alford, 2012) and by extension, on living hosts should not be underestimated. We report on an outdoor mesocosm trial using naturally infected tadpoles treated with bacteria that is already established on surviving adults in the study populations.

METHODS

Animal collections

In May 2011, two hundred and forty tadpoles of *A. obstetricans* were collected from four distinct *Bd*-positive sites regions within Switzerland: a forest pond in Zunzgen (Z) found in northwestern Switzerland's canton Baselland, a shallow garden pond in Altstätten (A) found in northeastern Switzerland's canton St. Gallen, a well-maintained garden fountain pond in central Switzerland's canton Luzern (L), and an open-meadow pond in Gamlikon (G) found in central Switzerland near Zurich. Ponds were found at a similar altitude ranging from 483-600 masl. Criteria for selecting pond sites were: confirmed *Bd*-positive in previous surveys (Garner *et al.*, 2005; Tobler *et al.*, 2012; Tobler & Schmidt, 2010) and harboring large populations of overwintering tadpoles that had not yet reached Gosner (1960) Stage 42 (i.e. with denticles still intact), permitting capture of sixty tadpoles in a single sampling. Collection permits were provided by the Nature Conservation Offices of the cantons Basel, Luzern, St. Gallen, and Zürich. Animal procedures were approved by the Veterinary Authority of Zurich (permit number 227/2007) and the Federal Office for the Environment. Uninfected metamorphs were returned to their pond of origin upon completion of the experiment according to permit guidelines. Standard field equipment disinfection protocols were adhered to during fieldwork and in-between site collections (Schmidt *et al.*, 2009).

After collection, tadpoles were temporarily stored in 20 L tubs in the lab at 19 degrees Celsius constant in order to confirm infection status using rt-PCR according to Boyle *et al.,* (2004) before being transferred at the same time point to the outdoor semi-natural mesocosms for the probiotic trial.

Bacterial isolation and probiotic therapy

Two bacterial strains, *Pseudomonas fluorescens* and *Flavobacterium johnsoniae* (EMBL Nucleotide Sequence Database Accession no. 70_d_1 and no. 76.5_c respectively), were originally isolated from the rinsed skin surfaces of tadpole and adult *A. obstetricans* from Itingen in Kanton Baselland, May 2009 and selected out of approximately 250 isolates collected from this species (Davis and Woodhams, *unpublished data)*. Isolates were originally screened for ability to inhibit *Bd* growth and capacity to resist *A. obstetricans* AMP secretions. We selected *P. fluorescens* based on: (a) high prevalence; isolated from 12 of 19 adult hosts examined in the survey, (b) potential to persist across generations and occur in amphibian hosts from Australia and from North and Central America (Lauer *et al.*, 2008; Walke *et al.*, 2011; Woodhams *et*

al., 2007) and (c) common implementation as a biocontrol in agriculture, due to a highly conserved, 2,4 DAPG antifungal metabolite, (Keel *et al.*, 1996). As probiotic inoculation would take place at the tadpole stage in this experiment, a second probiotic, *F. johnsoniae,* was selected because it was commonly isolated from *A. obstetricans* tadpoles. This common freshwater Gram-negative bacterium is also present in soil where it rapidly degrades chitin (McBride *et al.*, 2009) and certain strains have shown antifungal activity, a promising trait for a biocontrol agent against *Bd* (Sang & Kim, 2012). Cultures were prepared on low nutrient RIIA agar (BD Difco™) plates under a laminar flow hood and incubated for 72 hrs at room temperature. Ten plates per probiotic were each rinsed with 2 ml MiliQ water, incubated 5 min, and rinsed with an additional 5 ml MiliQ water and combined before treatment.

Experimental design

Using a standard protocol (Buskirk, 2002), a total of 48 mesocosm tubs (each 0.28 m^2, 80 L) were set-up on April 27, 2011 to contain 10 g of dried beech *(Fagus sylvatica*) leaf litter, 2 g of rabbit food, and 1 cup of diverse zooplankton in 60 L of aged tap water. A screen mesh protected tadpoles from direct sunlight and predator invasion. On May 16, groups of five tadpoles from each population were randomly distributed into 48 tubs organized in factorial design with the factors "population" (four levels, i.e. four populations) and "probiotic treatment" (three levels: one control and two bacterial probiotic treatments) four populations and three probiotic treatments. The treatment combinations of population-control were replicated six times whereas the treatment combinations population- *P. fluorescens* and population-*F. johnsoniae* were replicated three times.

After allowing tadpoles to acclimate to outdoor tub conditions for a week, all tadpoles from a mesocosms were collected with a tank-specific net into a sterile 1 L container filled halfway with water from the mesocosm and inoculated with either the probiotic bacterium or sterile medium by bathing the tadpoles for 2 hr in 2 mL probiotic slurry before being poured back into their mesocosms with the bacterial slurry. Groups of five tadpoles were inoculated with either *P. fluorescens* (n=12) or the sterile media control (n=12) on May 21, or with either *F. johnsoniae* (n=12) or sterile media control (n=12) on May 23. Control treatments consisted of rinses of sterile RIIA agar plates with MiliQ water.

Approaching metamorphosis (Gosner stage: 44), tadpoles were collected with a tank-specific net and housed in an autoclaved 1 L plastic tub containing 200 mL of the mesocosm's tub water and several beech leaves for shelter. These tubs were arranged

on shelving in keeping with the block design and rotated 2x weekly within the respective block. All tubs were tilted to simultaneously provide land and water access for the late-stage tadpoles and terrestrial toadlets. As the five tadpoles within a tank approached metamorphosis, they were added to their respective tank to complete metamorphosis. Toadlets were fed with crickets dusted with calcium three times weekly and the water changed the day after feeding.

Ambient temperature conditions were recorded every 3 hours with four HOBO H8 data logger (Onset Computer Corporation, Bourne MA, USA), placed on two corners and two inner tubs to record water temperature of the tadpoles in mesocosms and air temperature of the developing metamorphs. The experiment was completed one week following the last individual's metamorphosis (Stage 46), on August 8.

Monitoring survival, *Bd* loads, and body mass

The response variables examined to compare effects of probiotics and population across development among surviving juveniles are as follows: *Bd* infection across development, body mass at stage 46, and survival and body mass of juveniles at experiment's end. Survival of late-stage metamorphs was monitored every twelve hours, in the morning and evening, until the last day of the experiment. Upon death, specimens were removed from shared housing with vinyl gloves and the water in the tub was changed immediately with freshly aged tap water so that contaminants from the sloughing skin would not affect the other toadlets in the tub. The specimen was swabbed, weighed, and stored in 90% ethanol. Individuals observed with evident symptoms of chytridiomycosis and impending mortality (lethargy, refusal to eat) were euthanized as humanely as possible by applying a small dab of 20% benzocaine to the ventral drinking patch (Baby Orajel©, U.S.A.).

Sampling of *Bd*

During the course of the trial, *Bd* was sampled at four time-points with a sterile plastic rayon swab (COPAN Italia, S.p.A., Brescia, Italy). Since infection with *Bd* among tadpoles is restricted to the keratinized regions of the denticles, swabbing at the first two time points was limited to the buccal cavities of tadpoles with denticles (i.e. pre-Stage 42) upon collection from ponds and one week following inoculations (May 28 & 30). For the last two time points (3 &4) of toadlets, we swabbed the skin surfaces of each individual upon completion of metamorphosis (Stage 46) and of all surviving juveniles were swabbed the final day of the experiment. The entire body surface was swabbed beginning with each of the hindlimb and forelimb digits, flanks, and the ventral/dorsal surfaces (10x each). Separate pairs of gloves and paper cups were used when handling

each tadpole or metamorph to avoid contamination. Bias in swabbing technique was reduced by having only a single swabber for all individuals and time points in order towards the consistent capture of microbiota. Swabs were stored at -20°C prior to DNA extraction with a QIAGEN DNeasy Blood & Tissue Kit (Hombrechtikon, Switzerland) according to the manufacturer's protocol. To obtain sufficient genetic material for analyses, buccal swabs of tadpoles with denticles (before stage 43) were pooled by mesocosm for extraction with the exception of the last two time points, with metamorphs at stage 46 being extracted individually.

Assessing *Bd* infection status

Bd infection loads were analyzed with real time-polymerase chain reaction (rt-PCR) with Boyle's et al. (2004) protocol for all DNA extracts with *Bd*-specific primers from Microsynth: ITS1 (5'-CCT TGA TAT AAT ACA GTG TGC CAT ATC TC-3') and 5.8s (5'-AGC CAA GAG ATC CGT TGT CAA A-3'). Protocol modifications include: running 50 cycles, repeating the analysis when samples run in duplicate yielded inconsistent SDs greater than 0.5, and the FastStart Universal Probe Master was acquired from Roche: Chytridprobe, 6-FAM-CGA GTC GAA CAA AAT- MGB (Roche, Basel, Switzerland). All runs were quantified based on zoospore standards acquired from EcoGenics (Zürich, Switzerland, 2010) and stored at -80°C.

Statistical analysis

We tested for differences in survival of post-metamorphic individuals among treatments and among populations using a general linear mixed model (GLMM) with binomial error distribution and a logit link function (Kéry 2010). Each individual tadpole/toadlet served as a data point. Because individuals within tubs were not independent, we used the tub as a random effect in our models. Models were conducted in RStudio 0.94.110. and fitted using the lme4 package (Bates & Sarkar, 2007). We used the following strategy to analyze the data. First, we used AIC (Akaike Information Criterion) to determine whether there was a need to fit an interaction term (population*treatment). To do so, we used maximum-likelihood in the lme4 function glmer() to fit a model with and without the interaction. Because there was no need to fit an interaction term (see Results), we then used REML in glmer() to fit a model with the main effects "population" and "probiotic treatment". In the third step, we asked whether there were any differences between the treatments with *P. fluorescens* and *F. johnsoniae*. Because there were no significant differences (all $p < 0.05$), we pooled the probiotic treatments with *P. fluorescens* and *F. johnsoniae* into a single treament "pooled probiotic treatment". That is, we tested whether the control differed from the probiotic treatment. This is equivalent to fitting a contrast (Gamlikon vs. Altstäten) vs. (*P.*

fluorescens, *F. johnsoniae*) (Crawley, 2007).

We tested for differences in infection intensity for an individual at metamorphosis as a function of treatment, population, and mean air temperature using a linear mixed-effects model (lme) with a normal error distribution. The mean air temperature among experimental blocks was calculated for each day and these means averaged for a week leading up to the metamorphosis of an individual. As *Bd* loads vary independently with sampling day and treatment and swabbing took place on separate days for n=24 tubs, for this analysis each probiotic treatment (n=12 tubs) was compared with the paired control groups (n=12). In both analyses we accounted for the mescosm tank unit as a random effect. Tests were performed in RStudio 0.94.110.

To examine the association of survival with *Bd* loads at the larval stage, mean tub survivals were correlated with *Bd* infection loads one week following treatment with probiotics. A 10x dilution factor was calculated for raw mean zoospores. We report individual purported *Bd* loads at all timepoints. Since swabs were pooled at the first two timepoints, the raw *Bd* loads were divided by the number of individuals sampled at a given time point (i.e. 5 tadpoles per tub). At the second time point, after treatment, some tadpoles had metamorphosed beyond stage 42 and lacked denticles, and consequently an infection surface for *Bd*. Swabs for these individuals were consequently not included in the pooled set; only swabs for tadpoles with intact mouthparts.

RESULTS

Survival post-metamorphosis

Toadlets in the probiotic treatment tubs survived better than toadlets in control tubs (Fig.1). Survival also varied among populations, ranging from 21% in the Altstätten individuals (12 of 58) to 42% survival (24 of 57) in Zunzgen individuals. AIC model selection that a model without the interaction treatment-by-population (AIC = 282.6) did describe the data better than a model with an interaction term (AIC = 291.7). The GLMM with the factors "population" and "probiotic treatment" (i.e, control, *Pseudomonas*, *Flavobacterium*) showed no significant effects (Table 1). The difference between the control and the *Pseudomonas* treatment, however, was almost significant (P = 0.0752). After confirming that there was no difference between the two treatments "*Pseudomonas*" and "*Flavobacterium*" (all P > 0.660), we pooled the two treatments into a single "pooled probiotic treatment" and tested for effect of treatment and population on survival. This GLMM showed a clear effect of probiotic treatment on survival (Table 1). The best-fit GLMER model suggests survival was a significant response to both

population and probiotic treatment (Table 1). Across all populations, overall raw survival means for each treatment group are as follows: 25.23% (28 of 111) individuals survived from the control group, 38.98% (23 of 59) in the *F. johnsoniae* group and 40.35% (23 of 57) of individuals in the *P. fluorescens* group. Overall survival also varied significantly among populations (ANOVA, $p<0.05$), ranging from a low mean of 21% in Altstätten individuals (12 of 58) to a mean of 42% survival (24 of 57) in Zunzgen individuals (Fig. 2a).

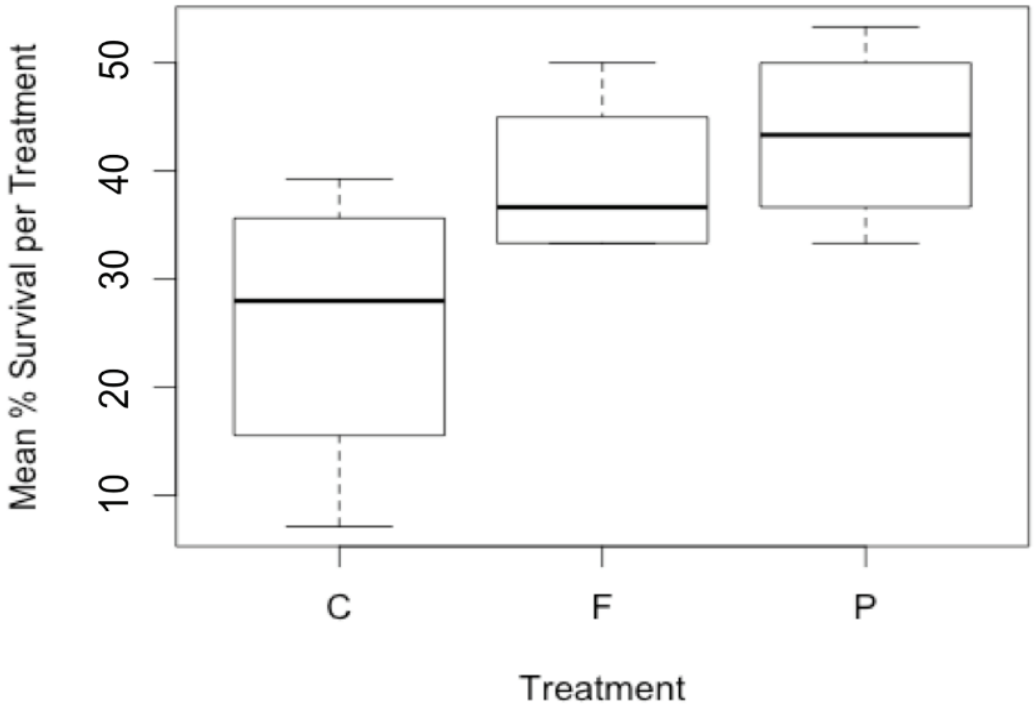

Figure 1. Boxplot showing mean (%) survival among mesocosms for 240 *A. obstetricans* toadlets in each of three treatments post-metamorphosis. Single-letter abbreviations on x-axis represent each treatment: control (C), and probiotics, *P.fluorescens* (F), *F. johns*oniae (F).

There was an association between survival and lower *Bd* infection loads upon metamorphosis of toadlets (Fig. SX1). Although there was no significant treatment-by-population interaction, an effect of treatment on survival rates is improved in all populations and treatments except for the Zunzgen *P. fluorescens* tubs where the survival rate is maintained (Fig. SX2).

Table 1. Results of best fit model for individual survival at experiment's end (1.1) and *Bd* infection intensities at Stage 46 (1.2) as a function of fixed effects: population, treatment, and (for loads) the mean ambient temperature in the week leading up to an individual's metamorphosis. All models account for mesocosms' tank as the unit. ANOVA tables follow; bolded *p* values indicate significance ($p < 0.05$).

Response	Parameter	Factor	Estimate	Std.Error	z-value	*p*	Model	AIC
1.Survival								
	Population	Altstätten	2.0044	0.4893	4.097	**<0.0001**	GLMER	282.6
		Gamlikon	1.4073	0.4341	3.241	**0.00119**	GLMER	282.6
		Schauensee	0.8956	0.4324	2.071	**0.03835**	GLMER	282.6
		Zunzgen	0.7247	0.4135	1.753	0.07965	GLMER	282.6
	Treatment	*F. johnsoniae*	-0.7367	0.4668	-1.578	0.11448	GLMER	282.6
		P. fluorescens	-0.8321	0.4634	-1.796	0.07253	GLMER	282.6
	Population	Altstätten	1.7495	0.3821	4.579	**<0.0001**	GLMER	285
		Gamlikon	1.2977	0.3457	3.754	**<0.0001**	GLMER	285
		Schauensee	0.7976	0.3347	2.383	**0.017163**	GLMER	285
		Zunzgen	0.6769	0.3213	2.107	**0.035123**	GLMER	285
	Treatment	Probiotics[a]	-0.6861	-0.6861	-2.276	**0.022874**	GLMER	285

Response	Parameter	Fixed Effect	numDF	F-value	*p*	Model	AIC
2. *Bd* loads at metamorphosis							
	Population	Population	3	60.16	**<0.0001**	LME	336.218
	Treatment	Treatment	2	9.46	0.1849	LME	336.218
	Temperature	Mean Temp[b]	1	1.73	**<0.0001**	LME	336.218
	Interactions	Pop*Trt	6	28.56	0.1301	LME	336.218
		Pop*Mean Temp	3	1.72	**0.0417**	LME	336.218
		Trt*Temp	2	2.89	0.6304	LME	336.218
		Pop*Trt* MeanTemp	6	2.52	**0.0289**	LME	336.218

[a] Probiotics pooled vs. Controls

[b] Mean temperature one week prior to an individual's metamorphosis Stage 46

Parameters for LME model fit by REML to describe Bd loads of individuals metamorphosis (n=70).

Bd infection loads across development and post- treatment

All tubs (n=48) containing a total of 240 tadpoles were confirmed *Bd* positive upon capture from their ponds in the wild. A week following bacterial therapy, there was comparatively no significant reduction in loads observed in the *P.fluorescens* treated tadpoles, although there was a reduction for the Altstätten-treated population (Fig. 2b). *Bd* loads in the *F. johnsoniae* tubs were significantly reduced for populations Schauensee and Altstätten, while the Zunzgen population observed comparatively higher loads than the control group (Fig. 2c).

Infection intensities varied among populations pre-treatment, post-treatment, and at stage 46; intensities leveled-out at experiment's end (Fig. 3). Zoospore loads were highest in wild-caught populations and linearly reduced over time under semi-natural conditions across all treatment groups until infections were nearly undetected in surviving juveniles. The exception is stage 46, where the greatest variation in *Bd* loads is observed (Fig 3). Of the four *Bd* sampling time points, three were synchronous, meaning all individuals within a population (1st time point) or within a treatment (2nd time point) and all surviving juveniles (4th time point) were swabbed on the same day to avoid bias in *Bd* load variation due to external differences in time and temperature. Asynchronous

sampling occurred at Gosner stage 46 (3rd time point) and thus depended upon on an individual's specific date of metamorphosis. As there was greater variation at this time point, *Bd* loads on a specific swabbing date (regardless of population and treatment) show a linear decrease in *Bd* loads at stage 46 from early June to early August (Fig. 4). Further, mean daily temperature in the week leading up to metamorphosis is plotted (Fig. 4) suggesting an inverse relationship between *Bd* load and temperature. Regardless of the asynchronous *Bd* swabbing at stage 46 due to individual dates of metamorphosis (and a consequent inability to control for the variability in time and temperature that may contribute to the variability observed) (Fig. 3) among individuals, the control metamorphs harbored twice the mean *Bd* load (59.2 zoospores) observed in the *P. fluorescens* (29.6 zoospores) and *F. johnsoniae* (28.7 zoospores) treatment groups (Fig. SX4). Our LME model suggests *Bd* load at Stage 46 is explained significantly by population, mean temperature leading up to metamorphosis, and a 3-way interaction of the previous two factors and treatment (Table 1.2).

While individuals at stage 46 observed significant differences in infection loads, individuals from different populations did not differ significantly by body mass (ANOVA is NS; Fig. SX5). Individuals from Altstätten had comparatively lower body mass and significantly lower survival compared than the other populations, supporting the impact of body mass on overall body condition (Tobler & Schmidt, 2010). There was also no significant association between body mass among treatments at metamorphosis or at experiment's end (Fig. SX5d).

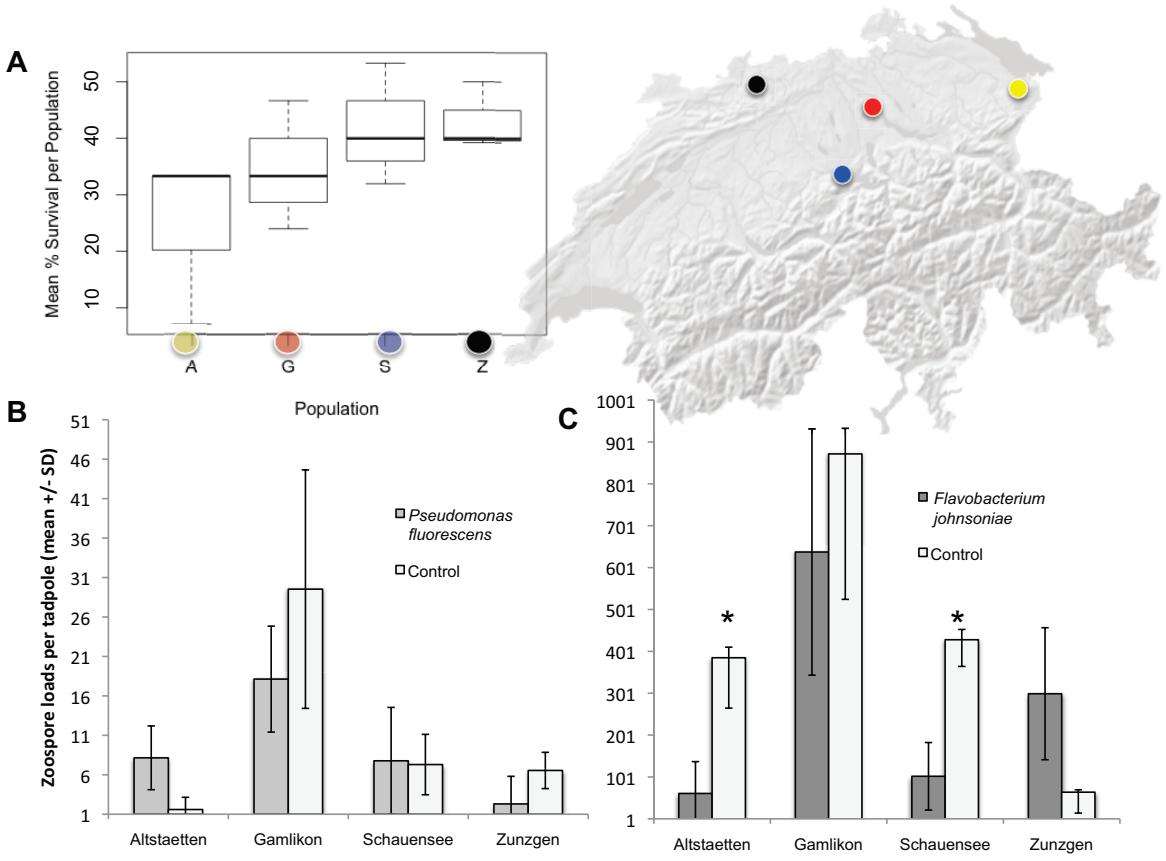

Figure 2. Boxplots showing among-population differences in: (a) mean-percent survival of toadlets post-metamorphosis and (b) change in mean *Bd* intensity (by raw zoospores) a week after probiotic treatments with *P.fluorescens* and (c) *F. johnsoniae*. Two populations, Schauensee and Altstätten saw significant (*) reductions in zoospore loads following *F. johnsoniae* treatment whereas Zunzgen saw a significant increase in B*d* loads. Topo map of Switzerland designates the four population sampling areas, represented by a yellow dot (Altstaetten), red dot (Gamlikon), blue dot (Schauensee), and black dot (Zunzgen). Map source: SwissTopo.admin.ch

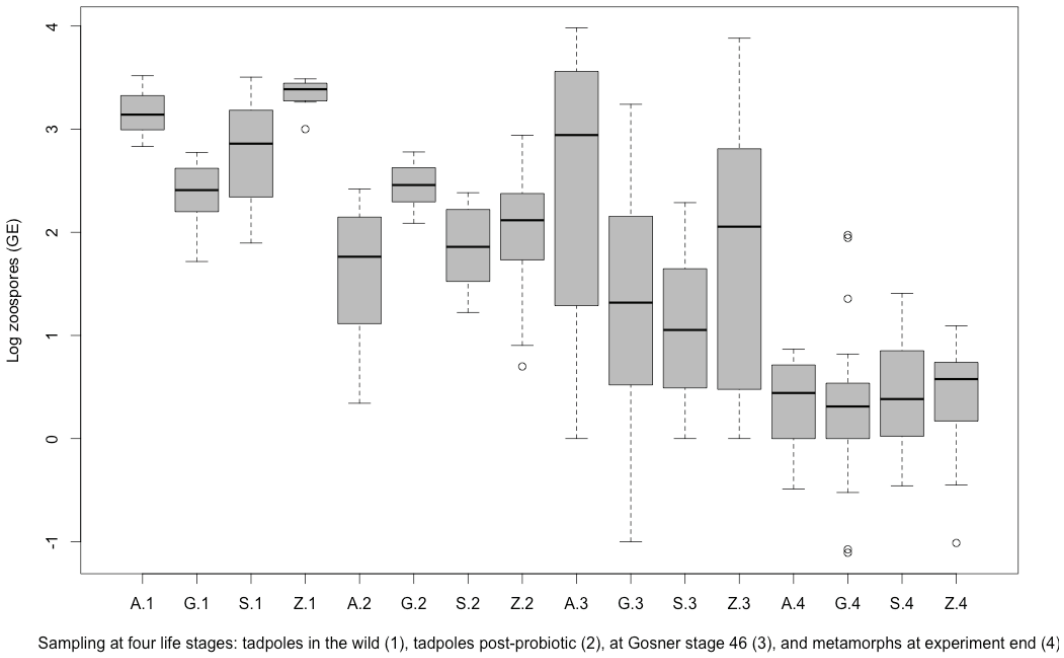

Figure 3. Boxplot of mean individual *Bd* loads by population at four sampling time segments: (1) in the natural ponds in the wild, (2) one week post-probiotic treatment, (3) at metamorphosis, and (4) at the end of Summer growing season. Standard deviations indicated by box-and-whiskers and outliers by hollow circles.

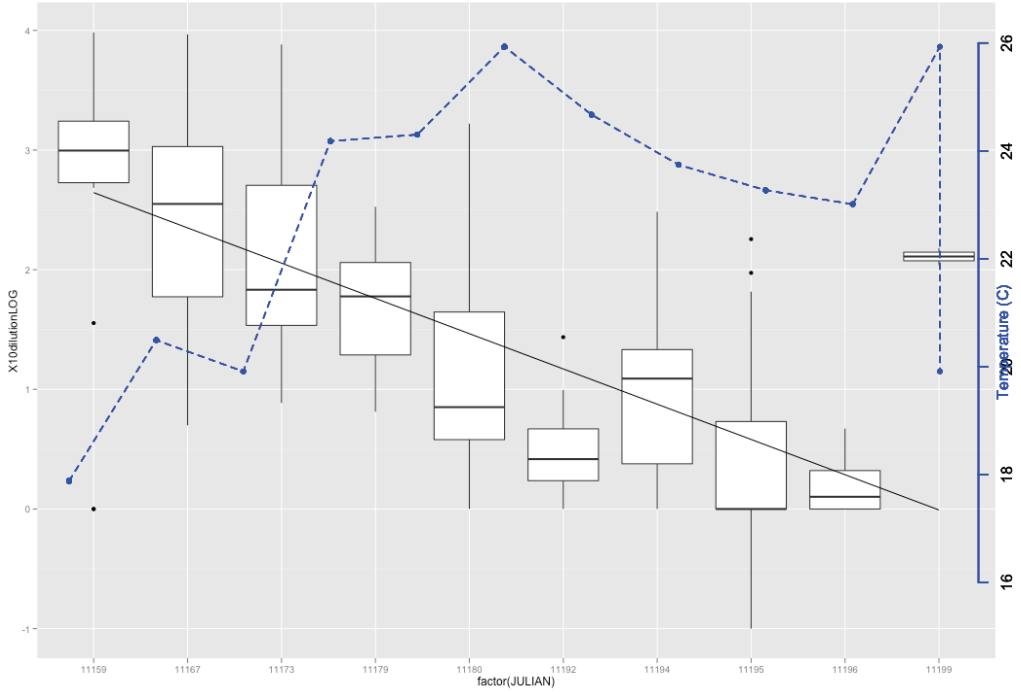

Figure 4. Spread of log-transformed *Bd* loads from the third sampling segment (seen in Fig.3) across time (Julian date), which varied with the day a toadlet reached Gosner Stage 46 June-August. Three-way interaction of treatment*population*temperature is significant. Standard deviations indicated by box-and-whiskers and outliers by black circles.

DISCUSSION

Microbiota are being incorporated into the host-pathogen-environment model of disease ecology (Scholthof, 2007) as either an extension of the host because they contribute to immune defense and extend the host phenotype (Woodhams *et al.*, 2007), or as an environmental component. Disease mitigation strategies including probiotics, bioaugmentation, and microbial remediation are increasingly used against diseases of medical, veterinary, and agricultural importance (Belden & Harris, 2007). Amphibian conservation biologists are also applying these strategies (Bletz *et al.*, 2013), although all previous published trials have been on adult amphibians and conducted under fixed laboratory conditions with the exception of a promising field trial on adult frogs (Vredenburg *et al.*, 2011). Here we considered the disease ecology system in the midwife toad, *A. obstetricans*, and the fungal pathogen *Bd* that infects the tadpole stage and causes morbidity upon metamorphosis (Tobler & Schmidt, 2010). We determined treatment at the tadpole stage as a critical time point in terms of feasibility and in preventing population declines of this threatened species in Switzerland.

Following a one-time probiotic treatment under semi-natural conditions, we found increased survival through metamorphosis of tadpoles naturally infected with *Bd*. The probiotic bacterium *P. fluorescens* significantly improved survival, while *F. johnsoniae* caused an initial decrease in the intensity of *Bd* infection, which did not lead to increased survival (Fig. 1). Considering host-microbiota symbioses have evolved over millions of years (Fraune & Bosch, 2010), the aim here was to augment a protective member of the microbial community found on the skin of healthy adults persisting in a *Bd*-endemic area. By treating hosts originating from four distinct populations we also targeted four distinct microbial community structures (Davis and Woodhams, *unpublished*). Deep sequencing of the microbial structures after treatment with a probiotic was previously shown to alter the microbial community structure as well as function (Davis and Woodhams, *unpublished*). Because there were no significant treatment-by-population interactions, most populations of this host species could benefit from probiotic therapy. However, a population-by-treatment effect (Fig. SX1) may be masked due to a limited sample size. Previous finding indicate a population-specific microbial community (Davis, 2013) and hence population-level differences may occur in terms of susceptibility to the pathogen, ability to assimilate a probiotic, and benefits conferred protective effects (Fig. 2b,c).

While mesocosms are not ideal surrogates for natural ponds (Diamond, 1986), trials are a crucial step in developing disease mitigation strategies and bridge a gap

between laboratory and field experiments (Winkler & Van Buskirk, 2012). For instance, there was an overall reduction in *Bd* loads of tadpoles from all populations after collection (Fig. 3) that could be interpreted as an artifact of bringing naturally infected tadpoles into mesocosms. We suggest this is more likely due to seasonal fluctuations in *Bd* burdens (Berger *et al.*, 2004; Conradie *et al.*, 2011; Kriger & Hero, 2006) from early to mid-Spring, a trend documented in natural populations of *A. obstetricans* tadpoles and correlated with rising water temperature (C. Geiger, *pers comm*). However, differences in survival rates among populations—from 25% in Altstätten to 43% in Schauensee (Fig. 2)—is consistent with previous observations of differential survival of tadpoles from distinct populations under controlled laboratory conditions (Tobler & Schmidt, 2010). Mortality rates of toadlets following metamorphosis can be astoundingly high, even in the absence of disease (Lampo & Giulio, 1998). Surmounting this survival hurdle is an important milestone towards improving a species' long-term survival by affecting population growth rates.

The mechanisms of increased host survival following probiotic treatments are largely unknown. Even common human probiotics such as *Lactobacillus spp.* do not have a clear mode of action nor do many widely used human drugs (Blaustein *et al.*, 2009). Several hypotheses for probiotics conferring health benefits include limiting pathogen growth while simultaneously enhancing commensal growth (Rosenthal *et al.*, 2011). Here, the expected mechanism is by reducing *Bd* loads, either directly by antifungal metabolites or indirectly through niche competition, preventing infection loads from reaching a lethal threshold (Carey *et al.*, 2006). Curiously, *F. johnsoniae*, unlike *P. fluorescens* was associated with reduced infection loads in some populations one week post-treatment (Fig. 2, Supplemental Fig. SX2). However, this trend did not lead to improve survival (Fig. 1). One explanation is the probiotic *P. fluorescens* may have taken longer that *F. johnsoniae* to establish and thus conferred benefits on the host later than the one week sampling point. Alternatively, *P. fluorescens* may not have disrupted *Bd* directly, but rather bolstered host health during the critical stage of metamorphosis. This could account for the significantly lower weights observed among survivors in the *P. fluorescens* treatment group at experiment's end (Fig. SX 5c), which could be puzzling as a greater percentage of these individuals survived better than individuals in the other treatment groups. As non-lethal weight loss is a common symptom of *Bd* infection (Davidson *et al.*, 2007; Tobler & Schmidt, 2010), this finding supports our observation for Altstätten, the population with lowest body mass (Fig. SX 5c) which is inversely associated with the highest *Bd* loads upon metamorphosis (Fig 3) and highest mortality (Fig 2).

It is unclear whether we can attribute survivorship to pathogen reduction or to a bolstered host immunity or condition that assisted the metamorphic transition. Regardless, our results at the empirical level obtained under controlled and randomized experimental conditions (Blaustein *et al.*, 2009) encourage mechanistic studies of this intriguing outcome. Historical examples of such empirical-based medical discovery whereby the mechanism may be elusive but the effect was clear and pronounced, date back to one of the founding probiotic thinkers, Ilya Metchnikoff. His theory of prolonged longevity among Bulgarian peasants who ingested fermented milk led to the lactic-acid producing bacteria, *Lactobacillus bulgaricus* (Metchnikoff, 2004). One future direction for evidence-based conservation (Sutherland *et al.*, 2004) in this trial will be to examine whether larva could pick-up the probiotics with specific primers to determine microbial loads for our symbiont before treatment, post-treatment, and at experiment's end (Becker & Harris, 2010).

How likely is therapeutic exposure of the host microbiota with a single bacterial isolate to influence the established microbial community in favor of host health? Both probiotics for this trial were carefully selected from a survey of the host's culturable skin bacteria and met *in vitro* screening criteria of pathogen inhibition and simultaneously withstand a natural mixture of *A. obstetrican's* antimicrobial skin secretions (Davis and Woodhams, *unpublished*). Probiotics were applied at a critical life-history stage just prior to metamorphosis. The probiotic must establish on hosts from different populations of origin and persist through the aquatic and terrestrial life-history stages. In a preliminary survey, both *F. johnsoniae* and *Pseudomonas spp*. were found on tadpoles, and both probiotics used in this study were abundant members of the cutaneous microbial community of adults in persisting populations. The adults were not infected with *Bd* despite the historical occurrence of *Bd* in this area (Davis and Woodhams, *unpublished data*).

Selected probiotics were also thought to be dominant members of the cultivable microbiota and were prevalent among wild hosts surveyed (Davis and Woodhams, unpublished). Bletz *et al.,* (2013) suggest that dominant or abundant microbial community members may be ideal probiotics if they also produce antifungal metabolites. *P. fluorescens* is a common symbiont across many taxa, isolated from amphibians in various parts of the world beyond Switzerland, including arboreal frogs in Panama (Walke *et al.*, 2011), the common pond frog *Rana catesbeiana* and the newt *Plethodon cinereus* in Northeastern USA (Culp *et al.*, 2007) as well as in amphibians in South America (Flechas *et al.*, 2012) and Australia (S. Bell, *pers comm*). In a survey of threatened *Rana muscosa* in the Sierra Nevada Mountains in California, *P. fluorescens*

was proportionally the most abundant and commonly-occurring isolate (Flechas *et al.*, 2012; Lam *et al.*, 2010; Woodhams *et al.*, 2007). There is evidence the symbiont is found to also occur in non-amphibious aquatic hosts including in the guts of zebrafish (Cantas *et al.*, 2012). As a broad-spectrum biocontrol agent implemented in agriculture crops (Brucker *et al.*, 2008), *P. fluorescens* is widely cited for its antifungal application via production of the metabolite, 2,4-diacetylphloroglucinol (=2,4-DAPG) (Lam *et al.*, 2011). However, no pathogen reduction could be deduced after a week in the tadpoles treated in this study. Thus, the purported mechanism linking to improved survival is unclear, yet the evidence-based widespread use of *P. fluorescens* maintains it could potentially prove a useful probiotic for other amphibians. For instance, *P. fluorescens* is not only uninhibited by our focal host's AMPs but has also been shown to be uninhibited by skin defenses of other hosts (Myers *et al.*, 2012). The success of a symbiont when applied directly to a tadpole under natural conditions is variable and underscores that selection of an optimal probiotic strain is critical and that outcomes of *in vitro* experiments may not correspond to intricate interactions present on the amphibian skin "medium."

In addition to host and microbiota, variation within pathogen factors can affect disease dynamics (Farrer *et al.*, 2011). Although we did not test for *Bd* strains present in our study populations, a previous study sampled tadpoles from Gamlikon and Zunzgen and found independent *Bd* strains, likely present in this trial: a global panzootic (widespread) lineage strain and a Swiss strain (M. Fisher, *pers comm.)* and yet, treatment with *P. fluorescens* elevated survival rates in both of these populations. Different *Bd* strains may be locally adapted to the microbiota, and context-dependency of the probiotics is also an important consideration because genes regulating antibiotic expression may depend on environmental temperature (Daskin and Alford, 2012; Woodhams and Brandt, *unpublished data*).

Future probiotic research must determine whether an applied probiotic successfully colonizes and persists on a host over time (Becker *et al.*, 2011) and whether it can be transmitted across generations (Walke *et al.*, 2011). Such studies should consider a healthy "microbiota baseline" against which to assess microbial community membership, structure, and function. The *Bd* pathogen may restructure the healthy microbiota into a dysbiotic (imbalanced) state (Bletz *et al.*, 2013) as documented in human periodontal, gut and bowel diseases (Sakamoto *et al.*, 2003). Whether protective bacteria operate by metabolic antifungals, immune modulation, or by niche competition remains unknown at this time. However, a single probiotic application to tadpoles can

significantly increase survival post-metamorphosis, and this outcome provides direction for future amphibian disease-management research.

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SUPPORTING INFORMATION

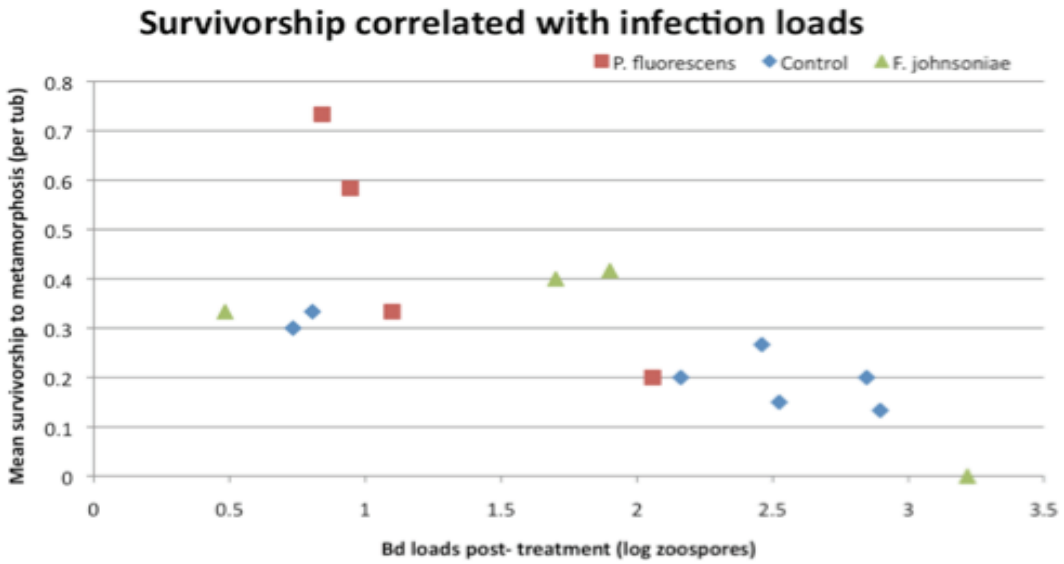

Figure SX1. Mean survival for mesocosm tubs correlated with tadpole *Bd* infection loads one week following treatment with *P. fluorescens* (square) and *F. johnsoniae* (triangle) and control. Higher survival rates are associated with mesocosms tubs with lower infection loads post-bacteria treatment.

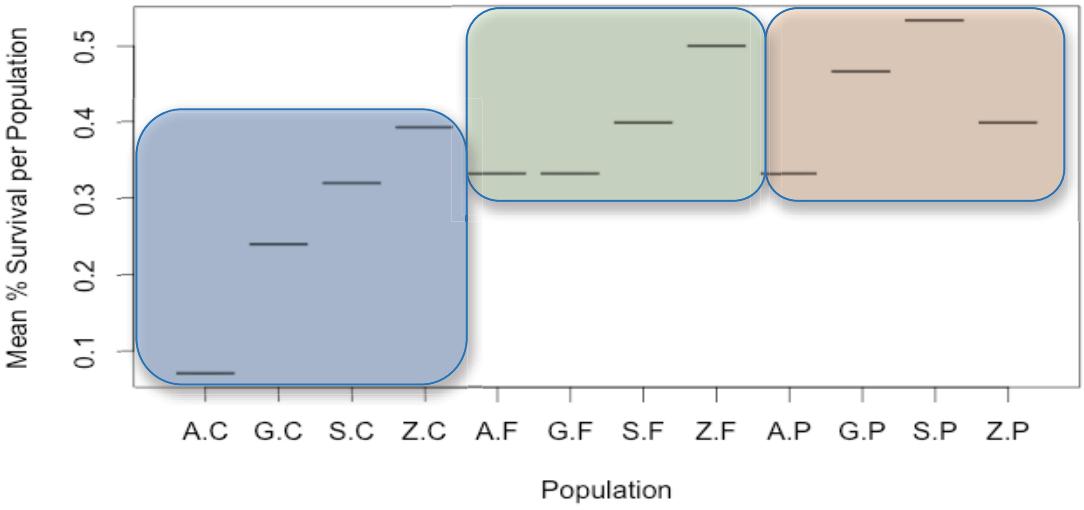

Figure SX2. Mean survival among mesocosm tubs (n=3), separated by population (A)ltstätten, (G)amlikon, (S)chauensee, and (Z)unzgen and by the treatment groups (C)ontrol, (F)l*avobacterium*, and (P)*seudomonas*, along the x-axis. Overall mean survival rates in control (blue-shaded populations) improved with probiotic therapies (green and red shading) for all populations except for Zunzgen, where survival mean was maintained in the *P. fluorescens* treatment.

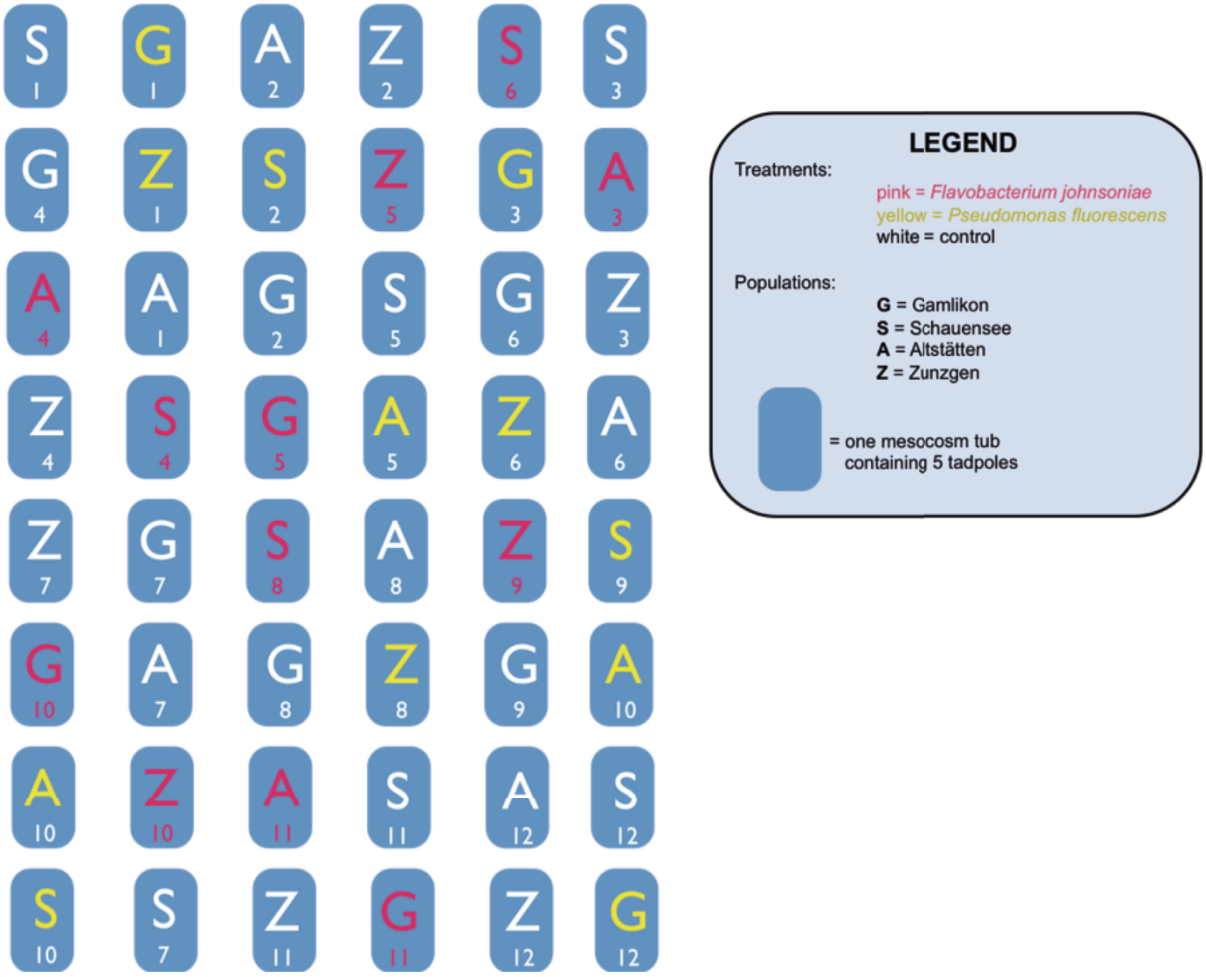

Figure SX3. Experimental design for raising and treating *A. obstetricans* tadpoles in mesocosms.

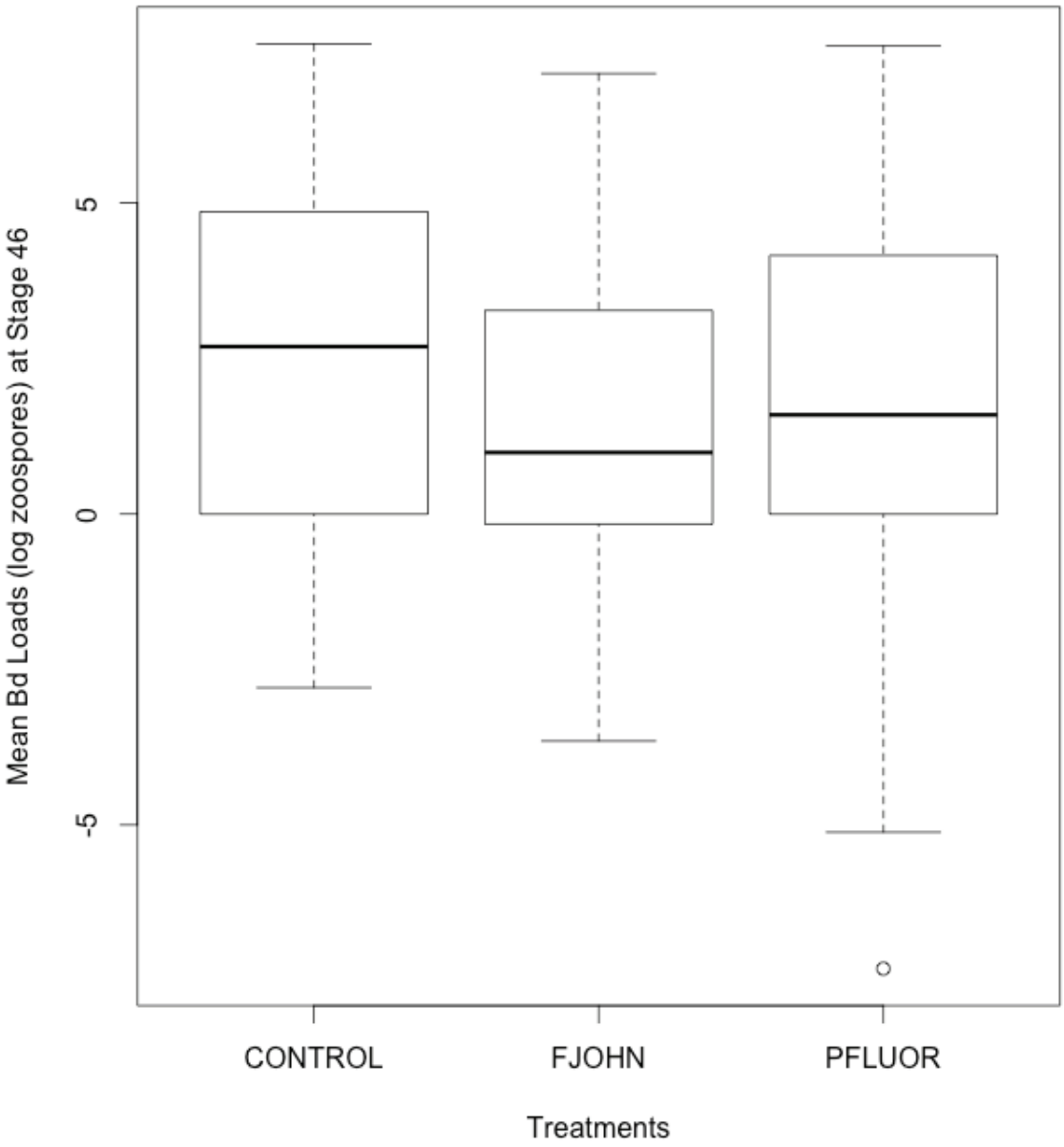

Figure SX4. Boxplots for log-transformed mean zoospore loads for individuals at Gosner stage 46 by treatment group. Zoospore burden, while lower in the probiotic treatments are not significantly different.

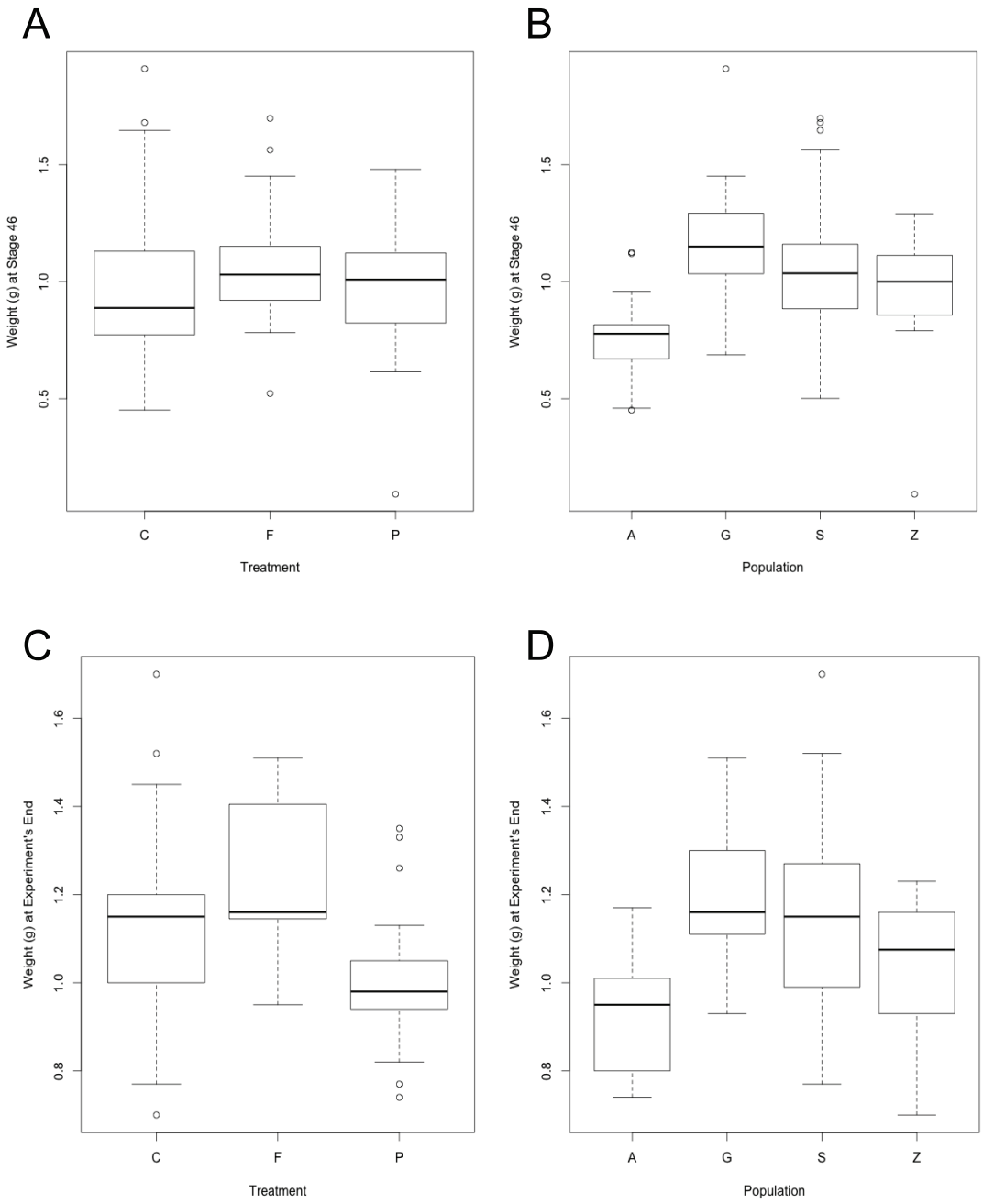

Figure SX5. Boxplots of juvenile body mass at Gosner stage 46 (A,B) and final day of experiment (C,D) by treatment and population. No significant population variation among treatment groups. Populations differ significantly in mean body mass at both sampling times.

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Publications

Daum JM, **Davis LR**, Bigler L, Woodhams DC. Infection, Genetics and Evolution Hybrid advantage in skin peptide immune defenses of water frogs (*Pelophylax esculentus*) at risk from emerging pathogens. *Infection, Genetics and Evolution.* 2012; 1854–1864.

Woodhams DC, Bosch J, Briggs CJ, Cashins S, **Davis LR**, Lauer A, *et al.,* Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Front Zool. 2011;8:8.

Working papers

Davis, LR, Bigler L, Woodhams DC. Trajectories of Amphibian Microbiota: Response to Biotherapy Depends on Initial Community Structure. (submitted to *ISMEJ)*

Davis, LR, JM Robertson, KR Zamudio, HL Rutschow, KJ Van Wijk, Q Sun, MM Haribal. Color morphs of the red-eyed treefrog, *Agalychnis callidryas* differ in skin peptide composition. (submission to the *Journal of Zoology).*

Research conferences and talks

Integrated Research Challenges in Environmental Biology (IRCEB): Emerging Wildlife Diseases threats to Amphibian Biodiversity - Arizona, USA (presentation), 2008-2011

International Bd mitigation workshop, Zürich, Switzerland (presentation), 2010

European Science Foundation "The Impact of the Environment on Innate Immunity: The Threat of Diseases" Obergürgl,- Austria (poster), 2009

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